Leaf spot and wilt of *Clematis* caused by *Phoma clematidina* (Thum.) Boerema.

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Clematis x 'Rouge Cardinal'

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Text and figure abbreviations

Ac	Acetone
AI	Active Ingredient
ANOVA	Analysis of Variance
^{13}C	Carbon 13
CBS	Centraalbureau Voor Schimmelcultures
CI	Chemical Ionisation
CMI	Commonwealth Mycological Institute
CUP	Plant Patholgy Herbarium, Cornell University
CV	Coefficent of Variation
ED_{50}	Median Effective Dose
EI	Electron Impact
FAA	Formalin Acetoalcohol
^{1}H	Proton
GDH	Glutamate Dehydrogenase
HPLC	High Performance Liquid Chromatography
k'	Capacity Ratio of Mobile Phase (see appandix 9)
LR	Linear Regression
LS	Longitudinal Section
LSD	Least Significant Difference
MBC	Methyl Benzimidazol-2-yl Carbamate
MIC	Minimum Inhibitory Concentration
MLP	Maximum Likelihood Program
NMR	Nuclear Magnetic Resonance
PAD	Instituto di Botanica E Fisiologia Vegetale, Universita di Padova
R_f	Distance Relative to Solvent Front
SE	Standard Error
SEM	Scanning Electron Microscope
t'	Peak Tailing Value (see appendix 9)
T5	0.05% (v/v) aqueous 'Tween 80'
TBA	Tertiary Butyl Alcohol
TEM	Transmission Electron Microscope
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
TS	Tranverse Section
UV	Ultraviolet

Graphs plotted with overall or mean standard error (SE) bar.

Plate abbreviations

All plates are calibrated by bar scales in microns. Unnumbered bar scales are 0.5 μm long, while numbered scales represent micron mutiples.

at	attachment point
ch	chloroplast
cl	collarette
co	conidiogenetic cells
cr	cytoplasm remains
cw	plant cell wall
dt	diseased tissue
fh	first hyphae
gm	granal matrix
gt	germ tube
ht	healthy tissue
hy	hypha
le	lesion edge
lo	conidiogenetic locus
ma	matrix
mt	mitochondria
mu	mucilage
nu	nucleus
os	ostiole
pl	plasmalemma
pw	pycnidial wall
py	pycnidium
sm	stoma
sp	spore
st	starch grain
th	thylakoid
tn	tonoplast
va	vacuole

n Number of replicates of each treatment/ factor combination

ABSTRACT

Phoma clematidina (Thum.) Boerema is identified as the causal agent of leaf spot and wilt of large flowered *Clematis* hybrids in New Zealand. Three distinct strains of the pathogen can be distinguished.

Phoma clematidina is a wound pathogen normally forming leafspots on both wilt susceptible and resistant cultivars. The final wilt symptom is usually the result of nodal rotting or "stem girdling" following hyphal extension into the node from the infected leaf, as *P. clematidina* is not a true vascular pathogen. Resistance to wilt was shown to be manifest by abscission or senescence of infected leaves.

Pathogenicity is associated with production of a toxin, ascochitine ($C_{15}H_{16}O_5$), which could be isolated from leaf lesions and culture filtrate. A reverse phase High Performance Liquid Chromatography system was developed to confirm purity of the ascochitine preparation before identification by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. Identification was confirmed by ^{13}C NMR spectroscopy and mass spectroscopy. Ascochitine induced endogenous electrolyte leakage from sensitive leaf cells and caused blackening of leaf tissue similar to that observed in leaf lesions. The main organelles affected appear to be chloroplasts and mitochondria although extensive cellular damage is evident.

Laboratory trials showed chlorothalonil and fenpropimorph to be the most effective fungicides in inhibiting *P. clematidina* spore germination and mycelial growth, and a mixture of these fungicides was used to effect control in the glasshouse. Cultural practices will however, remain an important factor in disease prevention and control.

CHAPTER ONE INTRODUCTION

1.1 THE CLEMATIS PLANT

1.1.1 Introduction

Clematis, a genus of the family *Ranunculaceae* encompasses approximately 250 species and numerous hybrids. The genus is of cosmopolitan distribution, but primarily concentrated in the temperate zones of both hemispheres especially Europe, India, China, Japan, North America, Africa and New Zealand. The generic name, *Clematis*, is derived from the Greek word for a vine branch, 'klema' (Jackman 1916). Anon (1970) credits Dioscorides with first use of 'Clematis' to describe a long branched climbing plant. The *Clematis* genus consists of climbers, perennial herbs and shrubs. Most plants, including the well known species and hybrids, are woody vines climbing by means of petioles adapted to twisting around suitable supports. *Clematis* species have been grown as ornamental plants since at least the end of the sixteenth century, proving to be very adaptable when cultivated. The natural habit for the climbers to run up through shrubs and trees, can be utilised to cover walls, porches, pillars, posts, trellises, arbors and pergolas, or as ground cover for the stumps of trees and mounds. The herbaceous species are often employed as border plants or grown as shrubs. Most species are deciduous, but the warmer climates do support less hardy evergreens (Lloyd 1977). Perhaps the best known clematis is *C. vitalba*, the only species native to England. This plant is popularly known as 'Old Man's Beard' because of the distinctive pale grey feathery styles, or as 'Traveller's Joy' whilst investing hedgerows and trees with large numbers of fragrant white flowers.

1.1.2 Flower, Fruit and Foliage

The *Clematis* flower is not composed of petals, but consists of petal-like sepals. Differences in flower morphology have been used by some systematic botanists to split two sections of the genus into two new genera, *Atragene* and *Viorna*. The *Atragene* group of which *C. alpina* is an example, has one or more rows of petal-like stamens between the sepals and the stamens. These are regarded as either petals or enlarged abortive stamens (Bean 1970). In the *Viorna* group, of which *C. viorna* is the type species, the sepals are joined at the edges to produce a bell shaped flower, while in most of the popular hybrids and species like *C. montana* the sepals usually spread out wide and flat. Both sections are usually regarded as part of *Clematis*; Bean (1970) and Bailey and Bailey (1952) treat the type species as belonging to *Clematis*, while listing the alternate name.

The achene, or seed vessel of clematis is terminated by a long style sometimes reaching 5 cm in length. The style is often covered by long silky hairs so that the fruit is often as striking as the flower.

The leaves are always paired at the node, 'and are occasionally simple but usually divided, consisting of the three, five, seven, nine, or perhaps fifteen leaflets' (Bean 1970). The leaves vary in form and size on the same plant, making descriptions difficult. While most *Clematis* species are hermaphrodite, nine species native to New Zealand are dioecious (Lloyd 1977).

1.1.3 Uses for Clematis

Clematis has been utilised for other purposes besides adorning gardens. The French name for *C. vitalba*, 'herbe aux gueux' (beggars plant) referred to beggars use of the acrid juice of the plant to induce ulcerous wounds which evoked pity (Bean 1970, Harper 1977). The juice of several *Clematis* species is acrid, as is the juice of many of the *Ranunculaceae*, causing erythema, blistering and sometimes dermatitis on contact with the skin. The Thompson Indians of British Columbia used a mild decoction of *C. ligusticifolia* and *C. drummondii* for the treatment of scabs and eczema, and as a headwash. A *C. ligusticifolia* decoction also found use as a tonic for general disorders despite the poisonous nature of the species (Anon 1970). The vesicant compound 'Protoanemonin', only reported in one *Clematis* species (*C. dioscoreifolia*) (Herz *et al* 1951), has shown antibiotic and anti-protozoan activity in laboratory trials (Holden *et al* 1947). 'Protoanemonin' is probably the active agent in the antibiotic activity reported for other *Clematis* species (e.g. Nickel 1959).

Some clematis have also found favour as a food. Lloyd (1977) quotes Thompson who wrote, 'I note in a French Encyclopaedia that clematis shoots are greatly esteemed for eating particularly by Italians and Russians'. Grieve (1931) in 'A Modern Herbal' lists *C. recta* as a herb, writing that the leaves and flowers when bruised irritate the eyes, throat and skin. Despite this, the herb was used for its diuretic and diaphoretic properties, and in the treatment of gonorrhea, syphilis, ulcers, cancer, eye infections and inflammatory conditions. Grieve also noted that a cure for itch could be obtained with a mild decoction of *C. vitalba*.

1.1.4 Diseases and Pests of Clematis

Clematis has long been regarded as easy to cultivate, suffering from few serious diseases. Besides wilt, which Lloyd (1977) considers 'is the most serious as to eclipse the rest', clematis is affected by several other fungal diseases. These include:

Powdery Mildew,

Erysiphe communis (Whitehead 1959)

Erysiphe polygoni (Moore 1943, Anon 1964, Anon 1970, Lloyd 1977, Pirone 1978);

Smut,

Urocystis carcinodes (Pirone 1978);

Leaf Blight,

Phleospora adjusta (Pirone 1978);

Rust,

Aecidium otagense (Allan and Zotov 1930);

Puccinia moriokaensis (Aecial state)
(Harada and Hasegawa 1975);

Puccinia okatamaensis (Aecial state)
(Harada and Hasegawa 1975);

Puccinia pulsatillae (Pirone 1978);

Puccinia recondita (Anon 1964);

Puccinia recondita var. *agropyri* (Pirone 1978);

Puccinia stromatica (Pirone 1978);

and Leaf Spot,

Cercospora rubigo (Pirone 1978);

Cercospora squalidula (Anon 1964, Pirone 1978);

Cylindrosporium clematidis (Pirone 1978);

Didymaria clematidis (Anon 1964);

Glomerella cingulata (Pirone 1978);

Phyllosticta clematidis (Pirone 1978);

Ramularia clematidis (Pirone 1978);

Septoria clematidis (Pirone 1978).

Crown gall caused by *Agrobacterium tumefaciens* is the only bacterial disease noted by Pirone. Lloyd (1977) notes that clematis is 'not officially subject to virus diseases' but suspects that viral diseases do exist. The symptoms he associates with viral disease include mottled and distorted young foliage and green and yellow mosaic patterns on expanded leaves. Anon (1964) reports a mosaic virus on Canadian plants of *C. ligusticifolia.*

All the common garden pests (i.e. slugs, snails, earwigs, scale, whitefly and aphids) are noted as attacking *Clematis* plants (Anon 1970, Lloyd 1977, Pirone 1978). Pirone lists two root-knot nematodes, *Meloidogyne hapla* and *M. incognita*, as pests as well as black blister beetle (*Epicauta pennsylvanica*) and clematis borer (*Alcathoe caudata*). Lloyd considers mice, rabbits, birds, deer and man as additional pests of clematis.

1.1.5 Clematis in New Zealand

Allan (1961) recognises 10 species of clematis (Puawhanga) native to New Zealand:

C. paniculata Gmel.

C. forsteri Gmel.

C. foetida Raoul

C. parviflora A. Cunn.

C. australis Kirk

C. australis var. *rutaefolia* Allan

C. hookeriana Allan

C. hookeriana var. *lobulata* Allan

C. petriei Allan

C. afoliata Buchan.

C. marata-quadribracteolata complex

All species are endemic and dioecious or polygamo-dioecious, with considerable hybridisation occurring between species. The introduced *C. vitalba* is considered a serious pest in native forests outgrowing and smothering native plants, while numerous other introduced species and varieties are propagated and growth for their ornamental value.

1.2 CLEMATIS HYBRIDS

1.2.1 Parent Stock

The first recorded hybrid was the small flowered *C.* x *Hendersonii* (now known as *C.* x *eriostemon* 'Hendersonii' (Lloyd 1977)) raised in England by Henderson in the 1830's (Jackman 1916, Russell 1933, Lloyd 1977). Whilst the cross may have been accidental, Lloyd (1977) and Bean (1970) consider that *C. viticella* and the herbaceous European species *C. integrifolia* were the parent stock, based on the hybrids' characteristics. However, it was the large flowered hybrids produced since the 1850's which have caught the imagination of horticulturist and general public alike.

Lloyd (1977) states that the large flowered hybrids are derived from three Far Eastern species and one European species respectively *C. florida*, *C. patens*, *C. lanuginosa* and *C. viticella.* A native of southern Europe, *C. viticella* was the first species introduced to England. Louden (a sixteenth century writer) attributes it's popular name, 'Virgin's Bower', as a compliment to Queen Elizabeth I, in whose reign it was introduced (Jackman 1916, Russell 1933). Considerable confusion exists as to the exact parentage of most hybrids and this situation is compounded when the authorities cannot agree on the status of the Oriental parent stock (Lloyd 1977, Bean 1970). *C. florida* and *C. patens* were both natives of China, but both have vanished from the wild. A cultivar of *C. florida* was introduced to Europe from Japan by Thunberg in 1776 but it was the Siebold cultivar of *C. florida* and the cultivated form of *C. patens* that were introduced to Europe by von Siebold in 1836 (Lloyd 1977). Thus at least two varieties of *C. florida* were available for hybridisation. Lloyd (1977) and Bean (1970) both note that *C. florida* is closely allied to *C. patens* and that some authorities unite them. Bean is quite specific in his descriptions of the difference between the two, definitely considering them to be separate species. This situation is further complicated by the status of *C. Fortunei* and *C. Standishii*, introduced to England by Robert Fortune in the 1860's (Jackman 1916, Lloyd 1977). Bean (1970) remarks that 'whether *C. florida*

is to be considered as one of the parents of the garden race of large-flower clematises depends very much on the status of *C. 'Fortunei'*...it is usually regarded as a variety of C. *florida* but is placed by Rehder under *C. patens*'. However, Lloyd (1977) thinks that 'if Fortunei is probably a derivative of of *C. patens*, Standishii is in the same boat'. Thus *C. Fortunei* and *C. Standishii* have been described as separate species, varieties of *C. florida* or *C. patens*, or the results of crosses between them. *C. Standishii*, originally a Japanese garden cultivar was probably used more often in hybridization than *C. patens*, though *C. patens* did make a significant contribution to the formation of the large flowered hybrids (Bean 1970, Lloyd 1977).

1.2.2 Hybridization

Lloyd (1977) considers that hybridization really started when *C. lanuginosa* was introduced to Europe by Robert Fortune in 1850. Issac Anderson-Henry of Edinburgh was the first to undertake systematic hybridization and in 1855 crossed *C. patens* with *C. lanuginosa* to produce *C.* x *reginae* (Jackman 1916). The largest hybrids resulted from crossing *C. patens* (and *C. Fortunei* and *C. Standishii*) with *C. lanuginosa*. Their main disadvantage was that the colours produced were pale like the white 'Henryi' or the mauve 'Lawsoniana'. George Jackman using *C. lanuginosa* as the seed parent produced, in 1858, the famous hybrid, *C.* x *jackmanii*. The pollen parent is thought to be *C. viticella* (Lloyd 1977, Bean 1970, Bailey and Bailey 1952) though some doubt remains as the pollen of both *C.* x *eriostemon* 'Hendersonii', and *C. viticella* 'Atrorubens' was used (Lloyd 1977). Whilst *C. viticella* contributes a rich deep purple colour to the hybrid, its considerable disadvantage was that the flowers obtained were smaller, never approaching the 23 cm span of a 'Lawsoniana' bloom.

While some of todays popular hybrids were bred just after the Second World War (e.g. 'Hagley Hybrid') most were bred during the period 1860-1880. Nurserymen like Jackman of Woking, Cripps of Tunbridge Wells, Morel of Lyons, Noble of Sunningdale and Smith of Worcester, raised many of the cultivars that are grown today (Jackman 1916, Russell 1933, Pennell 1965). These cultivars were often named after members of the nursery that raised the flower e.g. 'Jackmanii', or their patrons e.g. 'Lady Caroline Nevill'.

After the 1880's the excitement generated by hybridization died down. Lloyd (1977) wrote that the 'hybridists seem to have run out of ideas and anyway the epidemic nature of wilt disease put a damper on clematis cultivation.' There are few breeders actively involved with the breeding of new hybrids today. Lloyd (1977) is moved to state that 'the breeding story in this country is a bit pathetic'. However a few noteworthy varieties have been introduced to Britain from overseas including 'Dr Ruppel' from Argentina in 1975 and 'John Paul II' from Poland in 1981.

Although it is difficult to assign a cultivar to a particular group, the hybrids are segregated into three main groups based on the time of flowering and the general nature of growth (Bailey and Bailey 1952). The Florida group, whose flowers appear on

old wood in summer; the Patens group which flowers on old wood in spring and the Jackmanii group which flower on new wood in the summer and autumn. The Jackmanii group is further divided by some (e.g. Fisk 1977) into the Jackmanii, Viticella or Lanuginosa groups, based on differences in pruning requirements and flowering times.

1.2.3 Hybrid Propagation

The *Clematis* hybrids will not strike true from seed so are propagated from cuttings. 'Internodal cuttings', consisting of a pair of buds, one leaf, and approximately 5cm of stem below the node, are prepared from suitable lengths of stem. The cuttings, which can be dipped in a 'rooting hormone' (e.g. 'Seradix B2'), are pushed into a suitable rooting mix (e.g. 'Vermiculite') and bottom heat applied. Shading the cuttings, and watering especially during the early stages are essential, although moisture should not sit on the leaves for too long mainly to avoid *Botrytis* rot. After four weeks the cuttings should have rooted and can be potted into suitable containers and the plants allowed to harden.

In the United States 'nodal cuttings' consisting of two nodes with all but one of the upper leaves removed are preferred. These cuttings have the advantage that in American climatic conditions (hotter in summer, colder in winter) the cutting can grow from the lower buried buds if the upper pair dies (Lloyd 1977).

At New Zealand Clematis Nurseries in Christchurch, internodal cuttings are propagated in bottom heated shaded "forcing frames" situated inside glasshouses. Cuttings with one leaf removed are packed into 'Vermiculite' so that leaves do not overlap. Plants for sale and stock plants are grown or maintained in "shade houses". At the height of the growing season a large number of plants are closely packed in "shade houses" awaiting shipment or sale.

1.3 CLEMATIS WILT

1.3.1 Early Observations and Investigations

In 1872, Moore, writing in 'The Clematis as a Garden Flower' made no reference to pests or diseases of the cultivated clematis, but by 1885 Arthur reported a disease of clematis that 'threatened serious loss to all who hold either a pecuniary or aesthetic interest in it'. This disease attacked 'plants of all ages and conditions', and had 'almost stopped its propagation in some places...'. Arthur's collaborator, Professor Riley, found that the roots of diseased plants were swollen by a species of *Anguillula*, a nematode worm, but that the galls were not responsible 'for any marked deterioration of the plants'. Riley noted that a blackened rotting occurred at the crown of the plant, and Arthur investigating this aspect found that the roots at the point at which they arose from the stem were 'rotten for an inch or more'. This Arthur believed gave

'ample cause for the death of the foliage through cutting off the supply of nourishment'. Pycnidia containing 'many minute colourless spores' could be observed on the surface of very diseased roots, as well as internal mycelium and chlamydospores. Arthur stated that the pycnidial form 'belongs to the genus *Phoma*', but did not prove that the chlamydospores, pycnidia and mycelium were from the same fungus, though he suspected it. Arthur noted that *Phoma clematidis* Sacc. had been recorded on the stems of clematis plants in Europe but no *Phoma* species had been observed on clematis roots in the United States. Arthur finally concluded that 'one can only say that it is a pyrenomecetous fungus, growing within the roots and causing them to decay'.

Comstock, investigating 'the fatal clematis disease' in 1890, was convinced that the fungus simply accompanied the disease and that nematodes were the true cause. He noted that the most striking symptoms of the disease were that the leaves suddenly turned black and then the vine died back to a spot with a diseased appearance near the roots. He considered a species of *Heterodera* the agent of the disease writing, 'it is these worms cankering the roots of the plants that cause the disease'. Trelease (1885) and Klebahn in 1891 (cited Gloyer 1915a, 1915b) also considered that nematodes were the causal agent.

Bos, investigating 'decay in clematis' in 1893, observed that the 'affected plants have a diseased spot above the level of the ground; the lower parts are left in perfect health; this can be said in particular of the roots. The parts of the stem lying higher than the diseased spot remain uninjured at first; they dry up , however, because they can not get a sufficient quantity of sap'. He was able to repeatedly isolate a *Pleospora* species from the dead tissues, as well as species of non-parasitic *Anguillulids* (nematodes).However, he did not consider either to be the causal agent but suspected the disease was the result of mining injuries by an insect, 'for I discovered in the affected spots mines, which I could hardly consider to be the effect of the work of the nematoid worms I had found'. Bos eventually 'found on the affected spot, in the midst of the stem, a very small larva of a fly' and 'some brownish nymphae'. The nymphae hatched the little fly *Phytomyza affinis* Fall., 'which consequently must be considered to be the cause of the disease'.

E. S. of Woking wrote of 'the *Clematis* disease' in 1898, 'numerous experts on diseases of plants have studied the question, and the only reason they can assign is general weakness caused by excessive propagation'. E. S. felt that this reason was quite feasible, as he had never observed the disease in plants propagated by layering.

By the early 1900's wilt was so serious that many breeders were forced out of business. The French nurseryman, F. Morel gave up in despair and handed his collection of seedlings to William Robinson who, with his gardener Ernest Markham, created at Gravetye Manor an unrivaled collection of clematis in the 1920-30 period. Morel wrote of the disease in 1903, 'Un silence discret s'est fait dans la presse horticole sur ce mal redoutable et mysterieux, sans que celui-ci ait d'ailleurs interrompu ses

mefaits'. Morel thought the cause was bacterial, a view also held by Prillieux and Delacroix (1894).

Green, in 1906, reported on the susceptibility of *C.* x henryi and *C.* x jackmanii to 'blight', but did not attempt to ascertain the causal agent. Gloyer (1915a, 1915b) noted that Sorauer, in 1897, had attributed the death of infected plants to *Gloeosporium clematidis*, when he found 'gall-like' formations near soil level on stems of *C.* x jackmanii.

1.3.2 Gloyer's Investigation

In 1914, an abstract by W. O. Gloyer was published titled 'Stem rot and leaf spot of Clematis'. Gloyer had 'repeatedly found a slow-growing and probably undescribed species of *Ascochyta* on the leaves and stem lesions of the plants'. This is the first mention of leaf spot in the symptoms of the disease, and Gloyer was able to reproduce the disease symptoms by inoculating *C. paniculata* with a pure culture of the *Ascochyta*. In 1915, Gloyer established Koch's Postulates for the isolated fungus and described the causal agent as *Ascochyta clematidina* Thumen (Gloyer 1915a, 1915b).

Gloyer observed that plants were killed by growth of the fungus down the petiole into the stem thus girdling the plant at the node. Lesions could also develop internodally, resulting in a girdling of the stem at that point. He also noted that the disease manifested itself differently with respect to the various hybrids and species and whether the plants were glasshouse or field grown. In the glasshouse, both leaf spot and stem rot occurred, in the field only stem rot was observed, and on *C. paniculata* both symptoms were apparent.

1.3.3 Gloyer's Control Experiments

Gloyer investigated means of controlling the spread of *A. clematidina* in beds of *C. paniculata* in 1913-1914. A writer in 'Garden' (E. S. 1898) had stated that Bordeaux Mixture, when applied to diseased plants did not check the disease. Gloyer was able to demonstrate however, that Bordeaux Mixture (4-4-50 formula) when applied as a protective spray at fortnightly intervals, did significantly check the disease. A more effective protectant was soap and sulphur spray (1 lb soap, 6 lb sulphur, 15 gallons water) which when applied at a rate of 2.5 gallons per 150 sq ft, eradicated the disease. But spraying with soap and sulphur, or dusting with sulphur lead to acidification of the surrounding soil resulting in death of the *C. paniculata* vines. Gloyer found that *in vitro*, alkali-free Ivory soap 'at the strengths in which it is used as a contact insecticide has in itself fungicidal value...', but did not conduct field trials on the soaps protective properties.

Gloyer concluded that 'greater success can be attained by changing the methods of culture than by spraying', though 'spraying is beneficial to such plants, but before making such applications it is advisable to remove all diseased leaves and dead vines'.

1.3.4 Later Investigations in The Netherlands

It was not until 1963, that further attempts to control the disease were made. Blok (1963) demonstrated that under glasshouse conditions *A. clematidina*, *Diplodina vitalbae* (later reduced to synonymy with *A. clematidina* (Boerema 1979a)), and *Phytophthora cactorum*, could all produce wilt; however only *A. clematidina* was a pathogen in field trials. Blok found considerable variation in the time artificially infected plants of *C. jackmanii* took to wilt,and that neither maneb or chloropicrine were effective in controlling the disease. In 1965, *Coniothyrium clematidis-rectae* Petr. was first noted as a *Clematis* wilt pathogen (Blok 1965, 1966). This fungus has only been reported from The Netherlands as a clematis pathogen, and in that country plays a much greater role in the disease than *A. clematidina* (Boerema 1979a). Research in The Netherlands has concentrated on testing systemic fungicides for control of *Coniothyrium* wilt (e.g. Slavekoorde 1973, 1974, van Steekelenburg 1971, 1972a, 1972b).

1.3.5 The Wilt Situation in Britain: 1916-1966

Until 1966, *A. clematidina* was not regarded as the causal agent of clematis wilt in Britain. This situation arose from the belief, summarized by Whitehead in 1959, that 'it is not confirmed, however, the disease we know in Britain as wilt and American stem rot and leaf spot are one and the same...'. Neither Jackman (1916) nor Russell (1933) noted Gloyer's work when presenting their views on wilt. Jackman concluded that 'bacterial action' was responsible for 'sudden dying off' of clematis, while Russell firmly believed that 'excessive sun' was 'the primary cause of the die-back'. Markham in 1939 accepted that *A. clematidina* was the the causal agent of wilt, but felt that the disease was not always fatal as Gloyer had concluded.

Moore (1943) commenting on die-back of clematis noted that Gloyer 'considered most die-back was due to a fungus...*A. clematidina* Thum.', but concluded that in Britain 'the problem cannot yet be regarded as fully solved'. Moore listed 'bacteria, late spring frosts, overfeeding and bad grafting' as causes of die-back. He reported that 'die back apparently unrelated to *Ascochyta* was prevalent at one place in Som in 1936', but did note that 'a species of *Ascochyta*, agreeing closely with the description of Gloyer's fungus, was apparently responsible for die back in Devon (1937)'. A 'die-back' of clematis in 1939 originally attributed to *Ascochyta* was later found to be due to the toxic effect of chlorate contaminating the crocks (Hunt 1939, Moore 1943). Lloyd (1965) felt that *Botrytis cinerea* was the pathogen, entering the 'living plant tissue by way of a damaged or dead leaf stalk, of which there are always some at the base of a clematis stem, since the large-flowering types never shed their leaves cleanly', though Lloyd did accept that 'against this is the evidence that the fungus sometimes does its damage not at the node, but between nodes'.

In 1965, Ebben and Last working at the Glasshouse Crops Research Institute England, isolated a species of *Ascochyta* from wilted plants and established Koch's Postulates for this isolate (Ebben and Last 1966). They considered that 'Gloyer's

description of *Ascochyta* causing a leaf spot may be partly responsible for this fungus being overlooked as a cause of wilt. Leaf spotting of clematis has not been reported as such in this country'. However, Ebben and Last were able to isolate *Ascochyta* from leaf spots on eight *Clematis* varieties or species, thus establishing that leaf spot was a symptom of the disease in Britain.

1.3.6 The Current Wilt Situation

Some nurserymen and writers still do not accept *Ascochyta clematidina* as the causal agent and tend to confuse the issue by offering their own theories. Fisk (1977), whilst noting Gloyer's work, interprets Whitehead's (1959) comments as to leave 'Clematis Wilt unidentified with no clue as to the cause or cure'. Fisk offers his 'own personal pet theory...that it is not a disease at all but a failure of the very thin stem to cope with the sudden demand for moisture from the stem leaves and flowers which results in a breakdown of the tissues at a certain spot'. Fisk was still publishing a version of this theory in 1986 in 'Fisk's Clematis', his nursery's catalogue. Fisk's answer to wilt is 'to make sure they have a good supply of water at the base of the roots by giving them a good soaking two or three times a week'. Whitehead (1959) suggested 'empirical methods' to control wilt, as 'it has been observed that plants grown on their roots are less susceptible to wilt than those on grafted rootstocks...'. Jackman as early as 1916, had refuted this theory writing that 'grafting cannot be the cause...'. Russell (1933) and Markham (1939) also dismissed the idea that the stock (usually *C. vitalba*) 'overwhelms' the scion and causes its death. Jackman (1916) observed 'that within a few weeks after the plant has been re-potted it commences to form its 'own roots'... and is then mainly supported by them and not by the roots of the stock'. 'The public' still suspect that wilt is due 'to the nurserymans wicked practice of grafting' (Lloyd 1977), an idea nurtured by Harper (1977) who wrote, 'wilt used to be a serious problem but is less so now that most clematis are grown from cuttings instead of being grafted'.

Lloyd (1977) and Fisk(1977) considered that 'Benlate' (benomyl) when applied as a preventative spray or drench, does afford a degree of protection from wilt. Lloyd (1977) noted that a large flowered cultivar of *C. tangutica* was sensitive to Benlate (and insecticide); the foliage shrivelled although the plant was not killed. General plant hygiene is also considered important by most authors, cutting the infected stem well below the point of attack (Whitehead 1959, Lloyd 1977), painting the cut surface with fungus resistant paint and burning all diseased material (Whitehead 1959).

Wilt is responsible for large (*c* 50%) losses of some *Clematis* cultivars at New Zealand Clematis Nurseries in Christchurch, but less at the Gore Bay nursery (*c* 130km north of Christchurch) although exact figures are not available (Keay pers comm,* Lear pers comm*). Sunshine Environmentals nursery in Palmerston North, New Zealand* estimate only 5% of clematis are lost to wilt. No causal agent for wilt had been identified in this country nor any proven methods of controlling this disease elucidated. Overseas growers in Britain and the U. S. A. report only minor losses to wilt (Evison

pers comm,* Fisk pers comm,* Steffen pers comm*), attributing this to rigorous hygiene and use of fungicides.

(* see appendix 4 for addresses)

1.4 THE GENUS ASCOCHYTA

1.4.1 *Ascochyta clematidina* Thumen

Saccardo in his 'Sylloge Fungorum' lists four *Ascochyta* species found on *Clematis*:

A. clematidina Thum.

on living leaves of *Clematidis glaucae* (1884);

A. Vitalbae Br. et Har.

on twigs of *Clematidis Vitalbae* (1892);

A. indusiata Bres.

on leaves of *Clematidis erectae* (1899); and

A. Davidiana Kabat et Bubak

on dead leaves of *Clematidis Davidiana* (1906).

Gloyer (1915a, 1915b) 'repeatedly examined the species of *Ascochyta* on clematis and found it quite variable'. He felt that 'considering the variability of the fungus... any of the descriptions given for the different species of *Ascochyta* described on clematis would in general apply to it. Hence the name selected is the oldest one, *Ascochyta clematidina* Thum'.

Von Thumen's original description of *A. clematidina* was published in 1880:

Ascochyta clematidina Thum. nov. spec.- A. peritheciis epiphyllis, submagnis, emersis, dense gregariis, hemisphaericis, basi epidermide late cinctis, nigris in macula nulla vel obsoleta, ochracea, irregularia, subzonata, vix arescentia; sporis late fusoideis vel subellipsoideis, utrinque obtusatis, medio septatis, dilute fuligineo-griseis, 16-18 mm. long., 6-7 mm. crass.

Ad folia viva *Clematidis glaucae* Willd. in lapidosis pr. Minussinsk.-(no. 966.).

1.4.2 Form-Genera Classification

The form-genus *Ascochyta* was raised for those species of the Sphaeropsidales with two-celled spores found on the stems and twigs of vascular plants; similar fungi found on leaves were placed in *Diplodina*. Pycnidial fungi with one celled hyaline spores, collected from stems and twigs of vascular plants were assigned to the genus *Phoma*; those collected from leaves to *Phyllosticta*. The emphasis placed on the substrate and the presence of septa in the spore by the traditional Saccardoan system has recognised limitations when attempting to classify the deuteromycetes into form-

genera.(e.g. Guba and Anderson 1919). The difficulty of classifying a species that either occurs on both stems and leaves, or that produces one and two-celled spores from the same pycnidium, has been noted by many workers (e.g. Guba and Anderson 1919, Taylor 1941). This situation often resulted in one species having synonyms in all four genera. While some authors were 'forced to the conclusion that a number of the so-called species in genera like *Phoma*, *Ascochyta*, and *Diplodina* are nothing more than varieties of the same fungus' (Brooke and Searle 1921), others attempted to redefine the genera, but were 'naturally reluctant to relinquish such a convenient generic character as the presence or absence of a septum in the spores'(Dennis 1946). In 1936, Wollenweber and Hochapfel attempted to define '*Phoma*' species as having less than 5% septate spores and '*Ascochyta*' species as having 50-100% septate spores (cited Dennis 1946). This system still left those species with 5-50% septate spores as undefined (Dennis 1946). Rather than attempt to classify an isolate, some authors have adopted the term '*Phoma*-like' (e.g. Dorenbosch 1970), to describe isolates which would have traditionally been placed in one of the four form-genera: *Phoma*, *Phyllosticta*, *Ascochyta* or *Diplodina*.

1.4.3 New Concepts of *Phoma* and *Ascochyta*

In 1975 new definitions of *Phoma* Sacc. and *Ascochyta* Lib. were published (Boerema and Bollen 1975), based on electron microscope examination (Brewer and Boerema 1965, Boerema and Bollen 1975) and interpretation (Boerema 1965, Boerema and Bollen 1975) of spore ontogeny in several *Phoma* and *Ascochyta* species . Conidiogenesis was first utilised as a classification system for the coelomycetes to distinguish major taxonomic ranks by Sutton (Yokoyama 1985), with the emphasis for genera assignment shifted from conidioma morphology to conidial ontogeny. *Phoma* species were defined as showing phialidic ontogeny. One celled spores are usually produced *in vitro*, but secondary septation could occur, especially *in vivo* (0-95%). 'The first conidium is produced within a papillate protrusion of the undifferentiated parent cell; after a conidium secedes the basal part of the papilla remains as a collarette on the conidiogenous cell which may show two or three layers corresponding with the layers of the original papilla'. Species which showed annellidic ontogeny were placed in *Ascochyta*. 'The conidia arise as relatively thin-walled protrusions from undifferentiated parent cells'. The successive conidial primordia secede by a three layered septum. 'After or incidentally also before secession the conidia become two- (or more-) celled by invaginations of a newly produced inner wall layer (distoseptation). In this genus therefore conidial septation is an essential part of the conidium completion, which explains that *in vivo* as well as *in vitro* the conidia are always mainly two- (or more-) celled' (Boerema and Bollen 1975).

1.4.4 *Phoma clematidina* (Thum.) Boerema comb. nov.

On the basis of the above definitions, Boerema (1979a) renamed *Ascochyta clematidina* Thum. as *Phoma clematidina* (Thum.) Boerema comb. nov. after studying isolates from England, The Netherlands, and West Germany. He listed,

Ascochyta vitalbae Briard and Har. apud Briard;

Diplodina vitalbae (Briard and Har.) Allesch.; and

Diplodina clematidina Fautr. and Roum. apud Roum.

as synonyms. Boerema also noted that *P. clematidina* had also been isolated from cultivated *Selaginella* (CBS culture 520.66). Other *Ascochyta* species have been reduced to synonymy with *Phoma* species (e.g. Boerema *et al* 1965, Boerema 1972).

Many 'Phoma-like' fungi have been collected from clematis, but many descriptions do not state whether the plant was alive, diseased or dead at the time of collection. Only *A. clematidina* Thum. (von Thumen 1880), *Phyllosticta corrodens* Passer. (Allescher 1901), and *Phyllosticta intermedia* (Allescher 1901) were noted as having been collected from living leaves. Again considerable confusion is apparent in the classification of pycnidial fungi collected from clematis. *Phoma clematidis* Sacc. is described as occurring on both stems and leaves of clematis (Allescher 1901), while Briard (1891) described *Ascochyta vitalbae* Briard and Hariot as occurring on the dry twigs of *C. vitalbae.* Allescher (1901) corrected this "error" listing *Ascochyta vitalbae* Br. et Har. as a synonym of *Diplodina vitalbae* Br. et Har. found on stems of *C. vitalbae.*

1.4.5 '*Phoma*-Like' Plant Pathogens

Numerous plant pathogens are classified in the form-genera that comprise the '*Phoma*-like' fungi. Examples include *Ascochyta pisi* on peas, *Ascochyta fabae* on beans, *Phoma exigua* var. *exigua* on periwinkle (*Vinca minor*), *Phyllosticta antirrhinum* on snapdragon (*Antirrhinum majus*) and *Diplodina passerini* on Godetias. As noted previously the taxonomic difficulties associated with assigning a fungal species to a form-genus has resulted in numerous invalid generic determinations, and numerous synonyms as the fungi are reclassified (e.g. Boerema 1972).

1.5 TOXINS IN PLANT DISEASE

1.5.1 Historical Perspective

The toxin concept in plant pathology originated in the late 19th century with the beginnings of research into physiological aspects of plant disease. Anton de Bary is usually credited with the concept that toxins are involved in disease development when he applied sterile rotten carrot extract to healthy carrot tissue to reproduce a soft rot. There were further attempts to relate disease development to the toxicity of culture filtrates, but as early as 1926 Rosen questioned the definition of toxicity as it was

applied to compounds present in culture filtrates (cited Scheffer and Briggs 1981). It is now realised that the '... culture fluids of almost any microorganism are toxic to plants...'(Scheffer and Briggs 1981). Gottlieb (1943), to avoid the problems associated with the use of culture filtrates, extracted toxic fluid from the xylem of infected plants. While this approach is still controversial, 'Gottliebs' work was an important contribution...' (Scheffer and Briggs 1981).

Gaumann was 'the most vigorous proponent of the toxin hypothesis in plant pathology' (Scheffer and Briggs 1981). He stated 'micro-organisms are pathogenic only if they are toxigenic: in other words, the agents responsible for disease can damage their hosts only if they form toxins- microbial poisons- that penetrate into the host tissue'. Although this was regarded as 'a provocative statement for that time' (Graniti 1972), Gaumann defined "toxin" as all pathogen produced substances including enzymes (Graniti 1972).

The lack of a critical attitude in toxin research led to the postulates of Dimond and Waggoner (1953), and the term "vivotoxin" for 'a substance produced in the infected host by the pathogen and/ or its host which functions in the production of disease, but is not in itself the initial inciting agent of disease'. Graniti (1972) considered that the "vivotoxin" concept served 'to clarify the ideas of plant pathologists about toxins and plant diseases'. However with the realisation that any set of criteria was not reliable or satisfactory, the concept that 'each potential toxin must be considered on its own merits' has evolved. 'To establish a causal role in disease, each toxin must be examined by as many criteria as possible. No single criterion and no routine set of procedures is adequate' (Scheffer and Briggs 1981).

1.5.2 Toxin Definitions

The definition of the term "toxin" has evolved with the changing criteria for evaluating a toxin as a disease factor. De Bary, though credited with the conceptual idea that toxins are involved in plant disease, was actually investigating the effects of extracellular enzymes. Gaumann included 'some growth-affecting compounds' (Scheffer and Briggs 1981) and enzymes (Graniti 1972) in his definition of toxins. The descriptive prefixes (e.g. vivo, patho, host specific) added to the term to define a host or source have 'some limited uses, but can be confusing' (Scheffer and Briggs 1981). This is especially apparent when the term has different meanings in different scientific fields. A "mycotoxin" in human or animal pathology is a "phytotoxin" in plant pathology and an "antibiotic" in pharmacology (Graniti 1972). The definition of toxin employed in this investigation avoids the use of a confusing prefix to indicate a source or target organism. The definition used is that of Scheffer and Briggs (1981) '... a toxin is considered to be a microbial product other than an enzyme, which causes obvious damage to plant tissues, and which is known with reasonable confidence to be involved in disease development'.

1.5.3 Toxins of '*Phoma*-like' Pathogens

Phytotoxins have been implicated in disease and symptom expression of a number of '*Phoma*-like' pathogens. Leaf spots and lesions are the most common symptoms associated with '*Phoma*-like' pathogens. Other symptoms include damping-off (Guba and Anderson 1919, Boerema 1979b), wilt (Gloyer 1914, Taylor 1941), blight (Satya *et al* 1975, Paulson and Schoeneweiss 1971), and head lesions (M^{c}Donald 1964).

Phyllostine, isolated from the culture filtrate of a *Phyllosticta* species pathogenic on red clover, induced dark discolouration and wilting of red clover cuttings (Sakai *et al* 1972). Cytochalasin B (phomin) produces necrotic leaf spot symptoms on chicory (*Cichorium intybus*), potato (*Solanum tuberosum*), and periwinkle (*Vinca minor*) identical to symptoms produced by inoculation with *Phoma exigua* or application of diluted culture filtrate (Bousquet and Barbier 1972). Brefeldin A (ascotoxin, decumbin) has pronounced antibiotic, mycotoxic and phytotoxic properties and has been isolated from numerous fungi including *Ascochyta imperfecta*, *Phoma herbarum* and *Phyllosticta medicaginis* (cited Stoessl 1981).

1.5.4 Ascochitine

Ascochitine, described as a quinonemethide (Stoessl 1981) or benzopyran (Lepoivre 1982a), is a phytotoxic metabolite isolated from culture filtrates of *Ascochyta fabae* (Oku and Nakanishi 1963) and *Ascochyta pisi* (Bertini 1956), pathogens of broad bean and pea respectively. Ascochitine has also been isolated from *Ascochytula obiones* (Suter unpublished, cited Turner and Aldridge 1983), and from pea leaves infected with *Mycosphaerella pinodes* (= *Ascochyta pinodes*) or *Ascochyta pisi* (Lepoivre 1982b). Brown necrotic lesions on *Vicia faba* coleoptiles were induced by 10 ug/ml ascochitine solutions (Oku and Nakanishi 1963). Lepoivre (1982a) demonstrated sensitivity of pea (*Pisum sativum*) cultivars to induced electrolyte leakage by 100 ug/ml ascochitine solution was related to cultivar disease resistance. Lepoivre (1981) also related pea protoplast sensitivity to ascochitine with cultivar susceptibility to fungal infection.

Ascochitine is selectively toxic to higher plants, germinating plant seeds, bacteria, yeasts, germinating conidia and mycelial growth (Oku and Nakanishi 1963, 1966). The toxin induces membrane damage resulting in cell permeability changes and leakage of cellular constituents (Oku and Nakanishi 1966). Resistance to ascochitine by fungi is affected by toxin absorption rate and/ or possession of a biological reductive detoxification mechanism (Oku and Nakanishi 1966, Nakanishi and Oku 1969).

1.6 AIMS OF PRESENT INVESTIGATION

The principal aims of the present investigation were to determine the causal agent of *Clematis* wilt in New Zealand, study the reason for variable cultivar

susceptibility to wilt, and to investigate methods to control the disease. Several growers (Evison pers comm, Steffen pers comm, Fisk pers comm) felt the use of fungicides ('Benlate', 'Captan') gave effective disease control. Gloyer (1915a, 1915b) obtained disease control with a sulphur based mixture but soil acidification by the sulphur prevented continued use. Lloyd (1977) reported a clematis plant growing in sulphur dioxide impregnated soil did not suffer from wilt presumably because of the fungitoxic activity of this industrial pollutant. Ebben and Last (1966) suggested that fungicides sprayed to control clematis mildew may act to control the disease, while 'Bavistin' (carbendazim) is recommended for control of 'sudden decay' of clematis (Anon 1982). For these reasons a large number of fungicides were assayed *in vitro* for fungitoxic activity and a small glasshouse fungicide trial conducted.

Gloyer (1915a, 1915b) reported leaf spotting as a symptom of *Clematis* wilt, but this symptom is not generally recognised and had not been reported in New Zealand. Recognition of this symptom as part of clematis wilt in New Zealand, and investigations of disease development and progression in wilt susceptible and resistant cultivars were conducted to determine infection mechanisms and the basis for resistance.

A fungal toxin or enzyme was implicated in disease expression when preliminary studies of clematis leaf lesions indicated that hyphae was not present at the lesion edge. Gloyer (1915a, 1915b) had previously noted that mycelium were 3 to 5 mm from stem lesion boundaries. Ascochitine previously implicated in two *Ascochyta* plant diseases (Oku and Nakanishi 1963, Lepoivre 1982a, Lepoivre 1982b) was investigated for possible roles in disease expression, cultivar wilt susceptibility and isolate virulence.

CHAPTER TWO MATERIALS AND METHODS

2.1 PLANT MATERIAL

2.1.1 Clematis Stock

Plants of the cultivars 'Lady Betty Balfour', 'Ernest Markham', 'Rouge Cardinal' and 'Huldine' were purchased from Mr Alastair Keay of New Zealand Clematis Nursery, Christchurch. *Clematis montana* plants were a gift from Mr Ross Wilson of Christchurch.

2.1.2 Cultivation

2.1.2.1 Physical Environment. Plants were grown in an 'Exal' glasshouse constructed of 'Durolite', located in the grounds of the University of Canterbury. Temperature was automatically regulated by a 'Satchwell Climatronic CZT' glasshouse controller, operating a fan assisted heat radiator, a water cooled evaporation pad, and two extractor fans. Plants were grown on 0.9m high tables with tops slightly tilted for drainage. Plants were watered by two overhead sprinkler lines. Duration and time of watering were controlled by a preprogrammed irrigation controller ('Irri-Trol' MC-4/ MC-8) which was reprogrammed to account for seasonal requirements.

2.1.2.2 Pest Control. To control aphid, whitefly, and leaf-roller caterpillar, 'Ambush G' (permethrin), 'Shield' (triforine, acephate), 'Tedion V-18' (tetradifon), 'Malathion' (malathion) and 'Conqueror Oil' (mineral oil) were sprayed individually or in combination at the recommended rates as the need arose. 'Mesurol' pellets (methiocarb) provided effective slug control.

2.1.2.3 Potting Mix and Fertilizer. Plants were grown in a peat: sawdust: sand (4:3:2) mix containing 9 month 'Osmocoat' and other supplements (e.g. dolomite, potash) (Oderings 9 month potting mix) purchased from Oderings Nursery, Christchurch. Three month 'Osmocoat' was periodically applied to established plants.

2.1.3 Propagation

2.1.3.1 Forcing Frame. A 'forcing frame', constructed under a misting unit in a glasshouse, consisted of a sand bed in which a thermostatically controlled heating cable was laid, surrounded by 12cm high sides, and enclosed by a hinged lid covered with 50% shade cloth. The 'activator leaf' of the misting unit was placed inside the 'forcing frame'.

2.1.3.2 Plant Cuttings. Internodal cuttings were made from suitable lengths of *Clematis* stem. A sliver of bark was removed from the lower end of the cutting before dipping in 'Seradix B2' or 'B3' "root forming hormone". Cuttings were then pushed into "planter trays" of washed grade 2 'Vermiculite', and the trays placed in the forcing frame. For the first week extra shading was provided; after 3-4 weeks the lid was

opened so that rooted cuttings could begin to harden. Cuttings were regularly sprayed with 'Captan' (Captan 80; rate 5g/ 7l) to prevent *Botrytis* rot. After a further 1-2 weeks the cuttings were planted in peat mix in 9cm pots. Plants were transplanted into "planter bags" (diameter 16.5cm) after 3-4 weeks; a further 3-4 weeks growth was necessary before the plants were ready for experimental purposes

2.2 FUNGAL ISOLATES

2.2.2.1 Isolation

2.2.1.1 From Wilted Plants. A rotted node was placed in a "moist chamber". After 12h, cirrhi extruded by mature pycnidia were picked off with a sterile needle and placed onto GSP (glucose agar containing streptomycin and penicillin - see appendix 1). Alternatively small pieces of rotted tissue from inside nodes were placed onto GSP agar. Plates were incubated for 4-5 days at 20-25^{o}C.

2.2.1.2 From Leaf Lesions. Infected leaves were either placed in a moist chamber for 12-24h and the extruded cirrhi inoculated as above, or small pieces of infected tissue were cut from near the edge of the lesion and inoculated onto GSP. Plates were incubated as above.

2.2.2 Maintenance

2.2.2.1 Long Term Storage. After 4-5 days, small pieces of mycelium from the edge of a culture growing on GSP were transferred to potato dextrose agar (PDA (Difco)) slopes and incubated for 7 days at 25^{o}C. Slopes were either covered with sterile paraffin oil and stored at room temperature, or sealed and stored at 4^{o}C.

2.2.2.2 Short Term Storage. PDA plates inoculated with a core of mycelium were grown for 7 days at 25^{o}C, then stored at 4^{o}C. Seven day cultures were inoculated from these plates.

2.2.2.3 Reisolation. Periodically, cultures were inoculated onto *Clematis* leaf discs (section 2.5) and reisolated.

2.2.3 Type Cultures

Four isolates (201.49, 195.64, 102.66, 520.66.) of *Ascochyta clematidina* were purchased from The Centraalbureau Voor Schimmelcultures (CBS) (see appendix 4).

2.2.4 Herbarium Specimens

Dried herbarium specimens were examined and spore size measured.

From CMI (see appendix 4)

Ascochyta clematidina IMI 215890

Ascochyta clematidina IMI 269108

From CUP

Ascochyta clematidina Thum.: Barth., F. Columbiani 2503

Ascochyta indusiata Bres.: F. saxonici 1198

Diplodina Vitalbae (Br. & Har.) Allesch.: Sydow, Mycotheca germ. 620

Phoma herbarum West.: Krieger, F. saxonici 2431

From PAD

Phoma clematidis Sacc. 1267

2.3 FUNGAL CULTURAL STUDIES

2.3.1 Preliminary Studies

Fungal isolate LB was grown on four agar substrates (PDA (potato dextrose agar (Difco)), OA (oatmeal agar), GA (glucose agar) and MEA (malt extract agar) - see appendix 1) at four temperatures (15, 20, 25 and 30^{o}C) to determine the best combination for further experiments. Nine cm plastic Petri dishes containing *c* 30ml of substrate were inoculated with a 5mm core cut from the growing edge of a 7 day 25^{o}C PDA culture. Plates were incubated inverted in dark incubators and the diameter of the colony measured at regular intervals.

2.3.2 Isolate Cultural Studies

The five New Zealand isolates and the four type specimens were grown on PDA and GA at 20 and 25^{o}C as above.

2.3.3 *In Vitro* Spore Size

Spores from 7 day 25^{o}C PDA cultures were measured. The type cultures did not sporulate in culture, nor could they be induced to sporulate on *Clematis* leaf discs.

2.4 SPORE SUSPENSIONS

Spore suspensions were prepared for use as inocula for infection studies and for tests of fungicide efficiency.

2.4.1 Preparation

2.4.1.1 Method A. Ten ml of 0.05% (v/v) aqueous 'Tween 80' solution (T5) was pipetted onto a 7 day 25^{o}C PDA culture and the culture surface scraped with a sterile loop. The resulting suspension was strained through 4 layers of sterile muslin.

2.4.1.2 Method B. The suspension produced above was centrifuged at 3000*g* for 10 min and supernatant discarded. The spores were resuspended in 10ml of T5 solution, recentrifuged and resuspended in 2ml of T5 solution.

2.4.2 Glass Slide Germination

Appropriate spore suspensions were prepared in T5 solution and conidial concentration determined by haemocytometer counts. Drops of spore suspension (25*ul*) were placed on glass slides previously cleaned with detergent, rinsed in tap water and distilled water, dried and just before use cleaned with 95% ethanol. Slides were incubated in a moist chamber at 25^{o}C. After 24h germination was stopped by addition of a small drop of 10% lacto-phenol cotton blue (appendix 3) and spore germination assessed.

2.4.3 Assessment of Germination

A spore was considered to have germinated if the germ tube length was greater than half the diameter of the spore.

2.4.4 Germination in Growth Room

Conidial germination of spore suspensions (1x10^{6} conidia/ml prepared by method A) on glass slides in the growth room was assessed.

2.4.5 Germination on Leaf Surface

'Lady Betty Balfour' leaf discs (section 2.5) inoculated with spore suspensions of isolate LB were examined with the SEM (section 2.9) to check germination characteristics.

2.5 LEAF DISCS

2.5.1 Preparation and Environment.

Freshly harvested *Clematis* leaves were washed in distilled water and 16mm discs cut from the leaf blade with a core borer. Discs were floated abaxial side up in 9cm plastic Petri dishes at 25^{o}C (16h) day, 15^{o}C (8h) night cycle. Discs floating on distilled water and 50 *u*g/ml kinetin solution were visually assessed for senescence and general appearance after 7 days. As there was no visual difference, distilled water was used for all subsequent experiments.

Petri dishes were illuminated with 300 *u*E/m^{2}/s light intensity (*c* 20% full sunlight) when placed 0.6m beneath a bank of Phillips T2 65W/33 RS cool white fluorescent tubes supplemented with Atlas 15W incandescent bulbs.

2.5.2 Infection Studies

2.5.2.1 Spore Suspension. A spore suspension of 1x10^{6} conidia/ml in T5 solution produced by method A was used.

2.5.2.2 Effect of Wounding. Non-wounded leaf discs, discs with a single hole made by a sterile dissecting needle and leaf discs with the abaxial surface abraded for 1mm^2 with a sterile dissecting needle, were inoculated and assessed for infection.

2.5.2.3 Scoring of Infection. Leaf discs were visually assessed for infection beyond the point of inoculation and infection scored on a +/- scale. Diameters of developing lesions were measured regularly.

2.5.2.4 Sepal Infection. Sixteen mm discs cut from sepals of 'Lady Betty Balfour' were wounded, inoculated and assessed for infection as above.

2.6 GLASSHOUSE INFECTION TRIALS

2.6.1 Glasshouse Environment

Infection experiments were conducted in a glasshouse similar to that described in 2.1.2.1.

2.6.2 Establishment of Koch's Postulates

Koch's postulates were established with isolate LB on cultivar 'Lady Betty Balfour'. The axillary buds of the lowest node were removed with forceps and a mixture of mycelium and spores from a 7 day 25^{o}C PDA culture applied to the wound. Control plants with axillary buds removed had PDA applied to the wound. The pathogen was isolated and cultured from wilted plants as previously described.

2.6.3 Spore Suspension Inoculum

For all further infection experiments a spore suspension of 1x10^6 conidia/ml in T5 solution produced by method A (2.4.1.1) was used.

2.6.4 Leaf Infection

Wounded (by puncturing the adaxial surface of the leaf with a Pasteur pipette) and non-wounded leaves were inoculated with 50μl drops of spore suspension, 2h after the days last watering. Periodic assessment of infection was made, and lesion size measured.

2.6.5 Influence of Humidity on Infection

Gloyer (1915a, 1915b) found that plants sprayed with a spore suspension and kept under bell jars for two days, developed lesions on the third day. To ascertain the effect of glasshouse humidity on infection, wounded and non-wounded leaves on 'Lady Betty Balfour' plants were covered with plastic bags for 48h after inoculation with isolate LB and infection compared with uncovered leaves.

2.7 FUNGICIDE TRIALS

Fourteen 'protective' fungicides were assayed for inhibition of *in vitro* spore germination. Fourteen 'systemic' fungicides were assessed for *in vitro* activity against mycelial growth. Three fungicides, 'Ronilan', 'Saprol' and 'Sportak' were included in both trials as both properties were claimed by manufacturers. Fungicide minimum inhibitory concentration (MIC) values were used to compare fungicide effectiveness. ED_{50} values were not generally calculated. On the basis of these results 'Bravo' and 'Corbel' were tested for *in vitro* interactions, before assessment for glasshouse disease control.

2.7.1 'Protective' Fungicides

2.7.1.1 Fungicides Tested. The protective fungicides assayed are listed in table 2.7.1.

2.7.1.2 Assay Procedure. Spore suspensions of isolate LB were assayed against all 14 fungicides. A T5 suspension of $5x10^6$ conidia/ml was diluted to a final concentration of $1x10^6$ conidia/ml with fungicide solution so that the final active ingredient (AI) concentration of the fungicide was either 1000, 100, 10, 1, 0.1, or 0 *u*g/ml. It was assumed that the AI of the formulation was the only contributor to fungicide activity, and the "carrier" had no synergistic or antagonistic interaction with the active ingredient.

Drops of spore/ fungicide mixture (25 *u*l) were placed on clean glass slides (2.4.2) and incubated at 25°C in a moisture chamber. After 24h slides were scored for spore germination.

2.7.1.3 Extended Trials. All isolates were assayed against 'Bravo' as above, at final AI fungicide concentrations of 10, 1, 0.1, 0.01 and 0 *u*g/ml.

2.7.2 Systemic Fungicides

2.7.2.1 Fungicides Tested. The systemic fungicides assayed are listed in table 2.7.2.

2.7.2.2 Assay Procedure. All fungicides were initially tested against isolate LB. One ml of fungicide diluted in sterile distilled water was added to 99ml of agar, to give final AI concentrations of 100, 10, 1, 0.1, 0.01 and 0 *u*g/ml. The agar used for all tests was natural PDA (nPDA) (Appendix 1). Plates were inoculated, within 24h of pouring, with a 5mm core cut from the growing edge of a 7 day 25°C culture on PDA. Plates were incubated at 25°C and colony diameter measured periodically.

2.7.2.3 Fungicidal Activity. After 7 days, inoculum cores which had not grown were transferred to fresh nPDA without fungicide, and incubated at 25°C. After 7 days growth was scored on a +/- scale to determine fungicidal activity.

2.7.2.4 Extended Trials. 'Corbel' at final AI concentrations of 100, 10, 1, 0.1, 0.01 and 0 *u*g/ml was tested as above against all fungal isolates.

Table 2.7.1 Protective fungicides assayed

Chemical Class	Common Name	Trade Name	Formulation*
Aromatic	Chlorothalonil	Bravo	50 % F
	Dichlofluanid	Euparen	50 % WP
Dicarboximide	Vinclozolin	Ronilan	50 % SC
Dithiocarbamate	Mancozeb	Manzeb	80 % WP
	Maneb	Maneb	80 % WP
	Thiram	Thiram	80 % WP
	Zineb	Zineb	75 % WP
Elements	Basic Cupric Chloride	Copper Oxychloride	50 % WP
	Sulphur	Analytical	Sublimed
Heterocyclic	Iprodione	Rovral	25 % F
Imidazole	Prochloraz	Sportak	45 % EC
Phthalimide	Captafol	Difolatan	50 % F
	Captan	Captan	80 % WP
Piperazine	Triforine	Saprol	19 % EC

* Formulation: % AI by weight.

EC Emulsifiable Concentrate
F Flowable Liquid
SC Suspension Concentrate
WP Wettable Powder

Table 2.7.2 Systemic fungicides assayed

Chemical Class	Common Name	Trade Name	Formulation*
Antibiotic	Kasugamycin	Kausumin	5 % S
Benzimidazole	Benomyl	Benlate	50 % WP
	Carbendazim	Bavistin	50 % WP
		Delsene	75 % WP
	Thiophanate-Methyl	Topsin M	40 % F
Carboxamide	Benodanil	Calirus	50 % WP
Dicarboximide	Vinclozolin	Ronilan	50 % SC
Imidazole	Prochloraz	Sportak	45 % EC
Morpholine	Fenpropimorph	Corbel	75 % EC
	Tridemorph	Calixin	75 % EC
Piperazine	Triforine	Saprol	19 % EC
Triazole	Bitertanol	Baycor	50 % WP
	Penconazole	Topas	10 % EC
	Propiconazole	Tilt	25 % EC
	Triadimefon	Bayleton	5 % WP
	----------	BAS 9018	20 % EC

* Formulation: % AI by weight.

EC Emulsifiable Concentrate
F Flowable Liquid
S Solution
SC Suspension Concentrate
WP Wettable Powder

2.7.3 Fungicide Interaction

Some fungicides are reported to enhance the activity of another fungicide with a different mode of action (e.g. Gisi *et al* 1985). To test for such an interaction *in vitro*, 'Bravo' and 'Corbel' were assayed for spore germination inhibition, and activity against mycelial growth at all combinations of fungicide AI concentrations of 10, 1, 0.1, 0.01, and 0 *u*g/ml, using isolate LB.

2.7.4 Glasshouse Spray Trials

A mixture of 750 *u*g/ml AI 'Corbel' and 1500 *u*g/ml AI 'Bravo', was assessed for *in vivo* activity. Experiments were conducted in a glasshouse (2.1.2.1) with isolate HD and cultivar 'Huldine'.

2.7.4.1 Preventative Action. Plants were sprayed with fungicide mixture until run-off, 2h after the last glasshouse watering. Seven days later at the same time, leaves were wound inoculated with spore suspension and infection assessed at 7 day intervals.

2.7.4.2 Eradicative Action. Plants were inoculated, then sprayed 7 days later; disease expression and development were assessed as above.

2.8 OPTICAL MICROSCOPY

2.8.1 Preparation

Infected leaf discs were cut into small (4-5mm) pieces and fixed in FAA (formalin acetoalcohol - Appendix 2), dehydrated in a tertiary butyl alcohol (TBA) series, and infiltrated with 50:50 (v/v) TBA:liquid paraffin mixture. After further infiltration with wax ('Paraplast') the specimens were embedded. (For full details refer Appendix 3).

2.8.2 Sectioning

Serial sections (8*u*m) were cut with a glass knife on a 'Jung' rocking microtome, expanded on water and collected on glass slides previously cleaned with ethanol. The slides were then heated at 40^{o}C for 24h before dewaxing with 3 xylol washes.

2.8.3 Staining

2.8.3.1 Stains Investigated. Five stains were tested for their ability to differentiate fungal from plant tissue:

(1) Pianeze III_B

Malachite Green, Acid Fushsin, Martius Yellow

Formula and technique, see Gurr (1965) p294.

(2) Lepik's Stain

Safranin, Cotton Blue

Formula and technique, see Gurr (1965) p306.

(3) Ikata's Stain

Methyl-Violet, Eosin

Formula and technique, see Johansen (1940) p321.

(4) Stoughton's Stain

Thionin, Orange G

Formula and technique, see Gurr (1965) p316.

(5) Margolena's Stain

Thionin, Light Green, Orange G, Erythrosin

Formula and technique, see Johansen (1940) p320

The only stain that differentiated fungal from plant tissue was Pianeze III_B (see Appendix 3 for full details). Fungal and healthy plant tissue stained pink, while diseased or infected tissue stained various shades of green and yellow.

2.9 SCANNING ELECTRON MICROSCOPY

2.9.1 Sample Preparation

Small (*c* 30mm^2) pieces of leaf tissue were frozen in liquid 'Freon 12' before transfer to liquid nitrogen. Freeze-factured samples were broken under the surface of the liquid nitrogen. Samples were dried for 11h at *c* $2x10^{-1}$ Torr, then coated with 50nm gold in a 'Polaron E5000' sputter coater.

2.9.2 Examination

Stub mounted specimens were examined in a 'Cambridge Stereoscan 250 Mk 2' operating at 20kV. Photographs were recorded on 'Ilford FP4' ASA 125 negative film.

2.10 TRANSMISSION ELECTRON MICROSCOPY

2.10.1 Tissue Preparation

Small (*c* 10mm^2) pieces of leaf tissue were fixed in 3% (v/v) glutaraldehyde in 0.075M phosphate buffer pH 7.2, for 3h at room temperature (*c* 20^{o}C) under 2cm Hg vacuum. After washing with 3 changes of phosphate buffer for 1h, specimens were post fixed in 1% (v/v) OsO_4 in phosphate buffer for 2h at room temperature. Specimens were dehydrated in acetone at 15 min 20% stages, and 1h of 100% acetone (3 changes). Infiltration with 25:75 (v/v) Spurr's (1969) resin: acetone for 4h, and 75:25 Spurr's resin:

acetone for 12-16h preceded specimen embedding in 100% Spurr's resin. Blocks were polymerised overnight at 70°C (full details - appendix 2).

2.10.2 Sectioning

Ultrathin (*c* 80nm) sections were cut with glass knives on a 'LKB Ultrotome II system' ('LKB 4802A controller' operating a 'LKB 4801A ultrotome'), or by a diamond knife on a 'LKB 2128 ultrotome system'. Sections were collected on 'Formvar' coated 100 mesh copper grids or uncoated 400 mesh copper grids.

2.10.3 Staining and Examination

Sections were stained with 2% (v/v) uranyl acetate in 50% ethanol for 10min, and 'Sato's lead' for 5min (Hayat 1975). Sections were examined and photographed using a 'Hitachi HS-7S' electron microscope.

2.11 TOXIN ISOLATION AND PRIMARY PURIFICATION

2.11.1 Production

A liquid medium, modified from that described by Oku and Nakanishi (1963), developed to maximize toxin production contained: 20.0g D-glucose, 2.5g bacto-peptone (Difco), 2.0g K_2HPO_4, 1.0g KCl, 0.5g yeast extract (Difco), 0.1g $MgSO_4.7H_2O$, in 1000ml distilled water. Aliquots of 250ml sterilized broth in 500ml Erlenmeyer flasks were inoculated with two 5mm discs cut from the growing edge of a 7 day PDA (Difco) culture. The flasks were incubated at 25°C on a 'Gallenkamp' orbital shaking incubator (INR-200) at 50 (revs/ min).

2.11.2 Isolation

At appropriate time intervals, the contents of the flask were vacuum filtered through No. 54 Whatman filter paper, and toxin extracted from the filtrate. The residual mycelium was dried at 70°C for 8h and weighed.

2.11.2.1 Estimation of Crude Toxin Concentration in Culture Filtrate. A modification of the method of Oku and Nakanishi (1963) for ascochitine was employed. Culture filtrate (70ml) was acidified to pH 3.0 with 1N HCl, and 30ml chloroform added. This mixture was vigorously stirred, centrifuged for 5min at 3000*g*, poured into a separating funnel and left for 10min. After separation the solvent phase was evaporated *in vacuo* at 50°C and the crude toxin weighed.

2.11.2.2 Extraction and Primary Purification. The extraction procedure was developed from that described by Lepoivre (1982a). 500ml of filtered broth was acidified to pH 3.0 with 1N HCl and extracted with two 100ml aliquots of chloroform. The homogenate resulting from vigorous stirring was centrifuged, and then separated for 10 min in a separating funnel. Toxin was partitioned by stirring the solvent phase

with two 250ml aliquots of aqueous 0.4% (w/w) Na_2CO_3, and separating for 5min. After separation, the aqueous phase was adjusted to pH 3.0 with 1N HCl and extracted with 200ml chloroform. This mixture was funnel separated and the solvent fraction dried *in vacuo* at 50°C. The toxin preparation was washed from the flask with a minimum volume of methanol and centrifuged. The solvent was decanted and crystals air dried. This toxin preparation was used in biological assays.

2.11.2.3 Storage. Toxin crystals were stored dry in air, at room temperature in the dark as storage in chloroform, methanol or 0.4% (w/w) aqueous Na_2CO_3 resulted in discolouration of the solution.

2.11.3 Solubility

Ethanol, n-propanol, propan-2-ol, 2-methoxyethanol and acetone were visually assessed for ability to dissolve 1mg of toxin crystals (prepared by method 2.11.2.2), in 2ml solvent at 25°C. After the addition of 98ml distilled water to produce 2% (v/v) solvent solutions, the solutions were again visually assessed for toxin solubility.

2.11.4 Thin Layer Chromatography

2.11.4.1 Solvent systems.

(1) cyclo-hexane: chloroform: glacial acetic acid 7:2:1 (by volume)

(2) acetone: ethyl acetate: distilled water 5:5:2 (by volume)

2.11.4.2 Stationary phase.

(1) Silica Gel 1B 'Baker-Flex' (unactivated)

(2) Silica Gel B (Sigma) 0.25mm thick on glass (activated 1h 105°C)

2.11.4.3 Developing conditions.

Methanol solutions of extracts were applied to plates either as 10*ul* drops or a line of drops. Plates were developed for 1h in the dark, and visualized by:

(1) examination under normal light

(2) examination under 365nm monochromatic UV light

(3) exposure to sublimed iodine vapour

(4) sprayed with 70% (v/v) sulphuric acid and heated at 105°C for 20 min.

2.11.4 Toxin Production *In Vivo*

'Lady Betty Balfour' leaf discs were inoculated with isolate LB (2.5.2). After 7 days leaf discs with established leafspots were weighed and toxin extracted.

2.11.4.1 Extraction Procedure. Leaf discs were finely ground with a mortar and pestle in a minimum volume of distilled water. The volume was made up to 50ml with distilled water and pH adjusted to 3.0 with 1N HCl. Chloroform (25ml) was added, and the homogenate resulting from vigorous stirring was centrifuged. The solvent phase after funnel separation was extracted twice with two 50ml aliquots of 0.4%(w/w) aqueous Na_2CO_3. After funnel separation the aqueous phase was adjusted to pH 3.0

with 1N HCl and extracted with 25ml chloroform. The solvent phase after separation was evaporated *in vacuo* and the preparation weighed.

2.12 DEVELOPMENT OF TOXIN BIOASSAY

2.12.1 Toxin Assay System

The toxin produced by isolates of *Phoma clematidina* had chemical properties similar to citrinin and ascochitine. Induced electrolyte leakage by these two toxins has been reported by Betina and Barathova (1968) and Lepoivre (1982a), thus an electrolyte leakage assay was developed to quantify the effect of toxin solutions on *Clematis* leaf tissue.

2.12.1.1 Measurement of Conductivity. Conductivity of leaf tissue bathing solution was measured with a dip-type electrode connected to a 'Triac' conductivity meter. The cell constant of the probe had previously been calculated to be 2.68 after calibration against a solution of 0.01 dermal KCl (Anon 1978).

2.12.1.2 Toxin Solutions. Crystals were dissolved in acetone and slowly diluted with freshly autoclaved distilled water to a final solvent concentration of 5 percent (by volume). A concentration series was then prepared by serial dilution with 5% (v/v) acetone.

2.12.1.3 Plant Tissue. Leaf discs (16mm diameter) cut from mature *Clematis* leaves, were randomly selected and placed adaxial side down in glass M^{c}Cartney vials in 5ml of appropriate solution and submerged with an inverted Pasteur pipette. Each vial contained approximately 0.17g of leaf tissue. The discs were infiltrated by reducing the air pressure to about 2cm Hg for two 5min periods.

2.12.1.4 'Bathing Solution'. The infiltrating fluid was replaced with 10ml of fresh solution ('bathing solution'), and the vials incubated at 25^{o}C in the dark on a 'Gallenkamp' orbital shaking incubator (INR-200) at 50 revs/ min.

2.12.1.5 'Leaching Solution'. The discs were treated as above, but after 2h incubation the bathing solution was replaced with 10ml distilled water ('leaching solution') and reincubated as above. Elapsed time of the assay was measured from the addition of distilled water.

2.12.2 Determination of Assay Parameters

After 24h incubation the 'bathing solution' assay gave statistically significant results for electrolyte leakage from 'Lady Betty Balfour' leaf discs. Seven experimental parameters (see below) were investigated for this assay before the *Clematis* cultivars were ranked with respect to response to the toxin.

2.12.2.1 Solvent Effect. To estimate the interaction between solvent and toxin, leaf tissue was assayed against a toxin concentration of 10 ug/ml in 10% (v/v) acetone. The conductivity of the bathing solution was compared after 20h with results from a 5% solvent assay, to determine if the difference in overall conductivity could be attributed to a change in solvent response.

2.12.2.2 Effect of Time on Leaf Tissue Response. A definite time period was necessary to compare the effect of toxin concentrations on leaf tissue. Twenty hours was selected as this point was near the top of the linear part of all the net time response curves (i.e. overall-solvent response).

2.12.2.3 Response to Toxin Concentrations. The shape of the toxin concentration response curve was found by plotting 20h data from the time response curves. The sigmoidal shape obtained enabled recognition of the assay saturation concentration.

2.12.2.4 Assay Saturation Concentration. The 'plateau' of the dose response curve, representing saturation of the assay system, was found by a combination of visual curve fitting and statistical analysis of data.

2.12.2.5 Assay Minimum Concentration. Leaf tissue was assayed against 1 and 2.5 ug/ml toxin concentrations. Although the mean responses fitted the dose response curve, the responses could not be statistically separated from the solvent response. The minimum dose was therefore defined as that toxin concentration which gave a statistically different response from the solvent control.

2.12.2.6 Effect of Leaf Tissue Weight. Replicates of five leaf discs of 'Lady Betty Balfour' weighing 0.18g were used to develop the assay. A toxin concentration of 15 ug/ml induced greater electrolyte leakage from 5 'Montana' leaf discs (weighing 0.27g) than from 5 'Lady Betty Balfour' discs. Tissue response was therefore assayed in terms of equivalent leaf tissue weights rather than number of leaf discs.

2.12.2.7 Response of 'Montana' to Toxin Concentrations. The dose response curve for the resistant cultivar was determined to establish a toxin concentration or range of concentrations to rank the *Clematis* cultivars. The dose response curve also established that this assay could differentiate 'sensitive' from 'resistant' tissue.

2.13 TOXIN BIOASSAY

2.13.1 Cultivar Response to Toxin

Cultivars were ranked from most sensitive to most resistant in terms of toxin induced electrolyte leakage. Ranking, based on the net conductivity of the bathing solution after incubation of equivalent weights of leaf tissue with 5 ug/ml toxin solution for 20h, was achieved by calculating the Least Significant Difference (LSD) between mean pairs at t=0.05 probability level.

2.13.2 Mode of Action of Toxin

2.13.2.1 Assay. Five leaf discs (0.18g) of 'Lady Betty Balfour' were incubated with either 15 ug/ml toxin solution, solvent control or water control. After 20h small sections (3-4 mm^2) were cut from the discs and fixed for transmission electron microscopy.

2.13.2.2 Optical Observations. Four um sections of leaf tissue were cut with a glass knife and stained with Azur II or Pianeze III_B (appendix 3). Slides were viewed with a 'Leitz Orthomat' microscope and photographed on 'Kodak' Tungsten light film.

2.13.2.3 Transmission Electron Microscopy. Ultrathin sections cut from selected areas were stained and examined as previously described.

2.13.2.4 Effect of Cycloheximide on Induced Leakage. Cycloheximide inhibits biological reduction of ascochitine to dihydroascochitine by fungi (Oku and Nakanishi 1966). Leaf discs of 'Lady Betty Balfour' and 'Montana' were incubated in solutions of solvent control, $2x10^{-5}M$ cycloheximide, 5 ug/ml ascochitine and $2x10^{-5}M$ cycloheximide/ 5 ug/ml ascochitine as previously described to determine effect of cycloheximide on induced electrolyte leakage from *Clematis* cells.

2.14 ANTIBIOTIC SPECTRUM OF TOXIN

2.14.1 Test Organisms

(a) *Escherichia coli* (B46)[1]
(b) *Bacillus subtilis* (B45)[1]
(c) *Proteus vulgaris* (B 10)[1]
(d) *Staphylococcus aureus* (B50)[1]
(e) *Pseudomonas aeruginosa* (OT 296)[2]
(f) *Vibrio parahaemolyticus* (NLTC 10884)[3]
(g) *Candida albicans* (C 57)[1]

[1] Culture collection, Department of Plant and Microbial Sciences, University of Canterbury
[2] University of Otago
[3] National Health Institute, Wellington

2.14.2 Inoculum Preparation

Nutrient agar (Difco) (bacteria) or PDA (Difco) (yeast) slopes were inoculated and then incubated at 37°C (a,d,e,f,g) or 30°C (b,c). After 24h, 1-2ml of sterile distilled water was added, the slope shaken and 0.5ml of the resulting suspension spread onto previously poured Mueller-Hinton (Difco) agar in 9cm Petri dishes, and dried for 30min in a laminar flow cabinet.

2.14.3 Toxin Solutions

Toxin was dissolved in acetone and diluted with freshly autoclaved distilled water to a solvent concentration of 40% (v/v). A concentration series was prepared by serial dilution with 40% (v/v) acetone.

2.14.4 Assay Conditions

'Schleicher and Schull' 6mm antibiotic discs were saturated with toxin solution, excess solution drained and placed on plates seeded with the appropriate test organism. Plates were incubated inverted for 24h at 30 or 37^{o}C as above. Antibiotic activity was recorded as the diameter of inhibition zones around the assay discs.

2.15 TOXIN CHARACTERISATION AND IDENTIFICATION

2.15.1 Secondary Purification

For chemical identification the toxin preparation required further purification. The crystalline powder (2.11.2.2) was washed with a minimum volume of methanol, centrifuged and supernatant decanted. The powder was then washed with 10ml distilled water on a 'vortex' mixer, centrifuged, supernatant decanted and the powder dried in a desiccator. This preparation was stored under air in a desiccator, in the dark at room temperature.

2.15.2 Ultraviolet Spectrum

The toxin preparation obtained above was dissolved in spectroscopic grade methanol and spectrum recorded with a 'Pye Unicam SP 1800' spectrophotometer.

2.15.3 High Performance Liquid Chromatography

Toxin prepared as per 2.15.1 was dissolved in the appropriate solvent (methanol or acetonitrile) and eluted (see following conditions) on a 'Varian' Vista series 5000 Liquid Chromatograph equipped with a 'Varian' UV-100 detector.

2.15.3.1 Chemicals. Double glass distilled technical grade methanol, acetonitrile HPLC grade, acids analytical grade. Each component was separately vacuum filtered and degassed prior to use.

2.15.3.2 Elution systems.

(1) methanol: 0.8% (v/v) acetic acid

(2) methanol: 0.05% (v/v) trifluoroacetic acid

(3) acetonitrile: 1x10^{-2}M orthophosphoric acid

2.15.3.3 Columns.

(1) guard column 1.5cm RP18 7um pore 'Brownlee Labs'

(2) reverse phase 22cm C18 5um pore 'Brownlee Labs'

(3) reverse phase 10cm C18 5um pore 'Brownlee Labs'

2.15.3.4 Physical parameters.

(1) flow rate : 2 ml/min

(2) detector : 290nm

(3) mixture ratios: see results for details

2.15.4 Infrared Spectroscopy

Toxin infrared spectrums in fused KBr and 'Nujol' were recorded using a 'Pye Unicam SP3-300' infrared spectrophotometer.

2.15.5 Nuclear Magnetic Resonance Spectroscopy

Toxin ^{1}H and ^{13}C NMR spectrums in chloroform were obtained with a 'Varian XL-300' spectrometer.

2.15.6 Mass Spectroscopy

Toxin electron impact (EI) and chemical ionization (CI) spectrums were recorded by a 'Kratos MS80RFA' spectrometer.

2.16 STATISTICAL ANALYSIS OF RESULTS

2.16.1 Experimental Design

Experiments were designed (replicate blocking) so that replicate variation could be identified and accounted for during data analysis. Samples were randomly assigned to treatments to reduce experimental variation.

2.16.2 Analysis of Variance

Analysis of variance (ANOVA) was calculated by programs maintained on the Burroughs mainframe computer at the Computer Centre, University of Canterbury. Program output normally consisted of an ANOVA table with associated probability levels and 'Duncan's New Multiple Range Test' (DNMRT). This test does not assign confidence levels to differences between means, but assigns "protection levels" against finding false significant differences. Means linked by a solid line or with the same letter following or beneath them are considered equivalent at the probability level indicated (e.g. p=0.05).

2.16.3 Chi-Square Analysis

Frequency or contingency tables were analysed by the 'BMDP4F' program, which fits a log-linear model to each matrix and indicates probability levels for each factor effect and factor interactions.

2.16.4 Linear Regression and Maximum Likelihood

Linear regression equations, Pearsons moment correlation coefficient and probability levels for single x, multiple y data sets were calculated by computer programs maintained on the 'Burroughs' (see above). The Maximum Likelihood Program (MLP) which fits models to data was used to calculate fungicide ED_{50} values.

CHAPTER THREE RESULTS

3.1 FUNGAL ISOLATES

3.1.1 New Zealand Isolates

Fungal strains isolated from dead, wilted *Clematis* plants were designated with respect to the cultivar from which isolated i.e.:-

LB from *Clematis* x 'Lady Betty Balfour'

MT from *Clematis montana*

EM from *Clematis* x 'Ernest Markham'

HD from *Clematis* x 'Huldine'

RC from *Clematis* x 'Rouge Cardinal'.

3.1.2 Colony Characteristics

Colony size and colour were influenced by substrate and temperature. On PDA at 25°C isolates developed variable amounts of pistachio green (Rayner 1970) aerial mycelium by day 7 (figure 3.1.1). Brownish-tan pycnidia developed after *c* 4 days and by day 7 had dehisced hyaline, mostly one-celled spores in saffron-salmon coloured cirrhi. On GA there was little development of aerial mycelium by any isolate (figure 3.1.2). Browny-tan pycnidia developed from the fourth day and again dehisced hyaline, mostly one-celled spores in saffron-salmon cirrhi.

3.1.3 Type Cultures

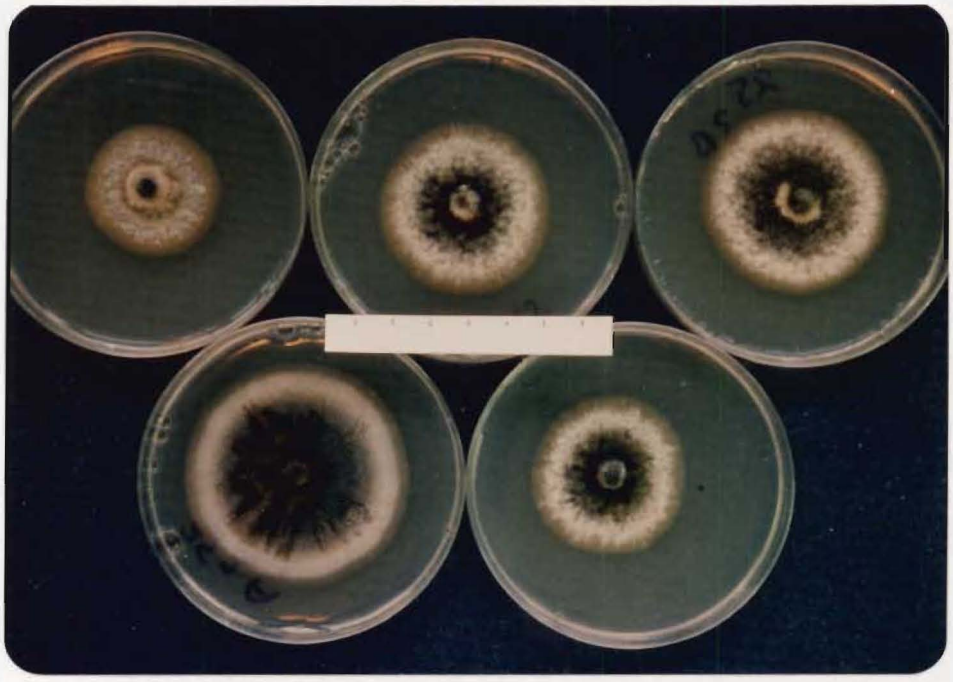
As noted previously type cultures of *Ascochyta clematidina* Thum. did not sporulate in culture. Various attempts to induce sporulation including inoculation on to wounded *Clematis* leaf discs and irradiation with near UV light were not successful.

3.1.4 Growth Characteristics

3.1.4.1 Preliminary Experiments. The effects of agar/ temperature combinations on colony diameter were compared after 7 days incubation (table 3.1.1). The best overall temperature for growth was 25°C. OA supported the fastest linear growth, followed by GA. However colonies growing on OA had low numbers of pycnidia and little aerial mycelium. When these factors are considered the best substrates were PDA and GA, so these were routinely used for further research. The optimum temperature for growth on PDA was 25°C, for GA 20°C.

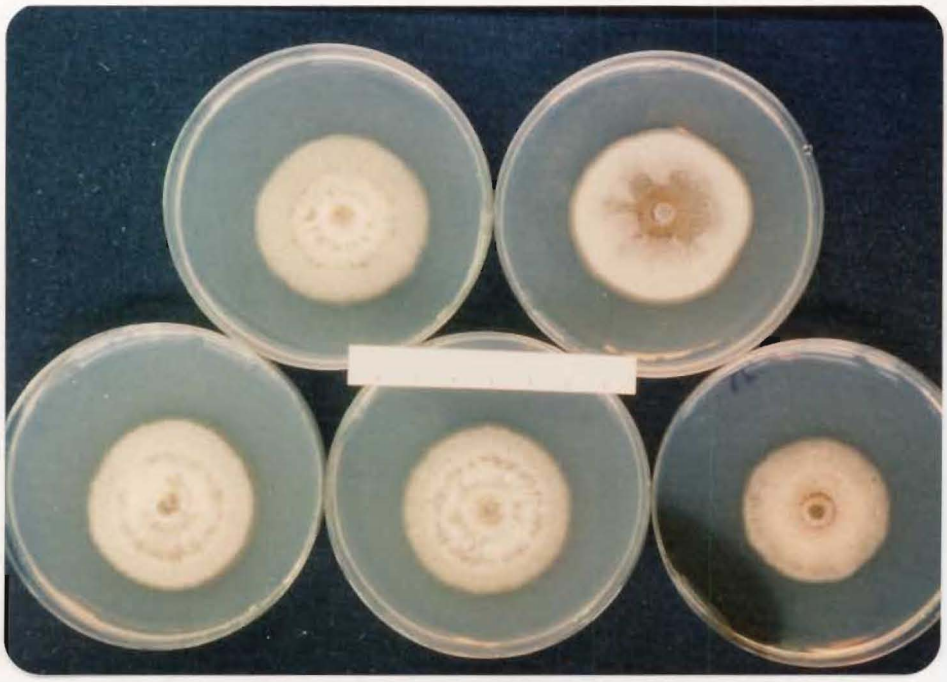
3.1.4.2 New Zealand Isolates. The five New Zealand isolates and four type cultures were grown on PDA and GA at 15, 20, 25 and 30°C. Three-way Anova analysis indicated significant ($p<0.001$) interactions between factors. Overall 25°C was the best temperature and GA the best substrate for linear growth, however overall

Figure 3.1.1 New Zealand isolates of *Phoma clematidina* on PDA



Isolate EM HD LB
MT RC

Figure 3.1.2 New Zealand isolates of *Phoma clematidina* on GA



After 7 days growth at 25^{O}C (scale bar = 1cm)

Table 3.1.1 Growth of isolate LB on different agars at four temperatures

Temperature (^{O}C)	Agar substrate			
	PDA	OA	GA	MEA
	mean diameter of 7 day colony (mm)			
15	25.8	40.6	32.5	21.2
20	41.7	56.8	51.0	33.3
25	50.8	57.3	46.3	43.3
30	29.1	42.0	38.7	31.9

CV = 4.23%

n= 6

Table 3.1.2 Growth of New Zealand isolates on two agars at various temperatures

Isolate	Substrate	Temperature (°C) 15	20	25	30
		mean diameter of 7 day colony (mm)			
LB	GA	32.0	47.3	46.0	37.7
	PDA	23.7	42.7	50.3	34.0
MT	GA	38.7	58.0	48.7	24.3
	PDA	42.3	65.0	57.7	25.3
EM	GA	22.3	37.7	39.0	31.3
	PDA	13.7	28.0	36.7	28.7
HD	GA	28.7	45.7	45.7	36.7
	PDA	19.0	34.3	43.7	34.7
RC	GA	29.3	43.7	45.0	38.7
	PDA	17.7	30.0	42.7	33.7

CV = 4.1% n=3

Table 3.1.3 New Zealand isolate association based on growth characteristics

	Isolate LB	MT	EM	HD	RC
Overall Mean[1]	39.2	45.0	29.7	36.0	35.8
DNMRT					
p=0.05				_____	
p=0.01				_____	

(1) colony diameter over all treatment combinations after seven days growth

Table 3.1.4 Mean colony diameter[1] after seven days of *P. clematidina* isolates

Isolate	Substrate	Temperature (°C) 20	25
102.66	GA	51.0	49.3
	PDA	50.7	62.3
195.64	GA	46.0	45.7
	PDA	47.7	51.7
201.49	GA	37.7	39.7
	PDA	29.7	28.3
520.66	GA	49.3	40.3
	PDA	49.7	54.3
LB	GA	47.3	46.0
	PDA	42.7	50.3
MT	GA	58.0	48.7
	PDA	65.0	57.7
EM	GA	37.7	39.0
	PDA	28.0	36.7
HD	GA	45.7	45.7
	PDA	34.3	43.7
RC	GA	43.7	45.0
	PDA	36.0	42.7

CV = 3.8% n=3

(1) diameter in mm

Table 3.1.5 Isolate association based on growth characteristics[1]

	Isolate								
	102	195	201	520	LB	MT	EM	HD	RC
Overall Mean[1]	53.3	47.8	33.8	48.4	40.6	57.3	35.3	42.3	41.8
DNMRT									
p=0.05		a			a			b	b
p=0.01	a	b		b	b		a	c	c

(1) colony diameter in mm after seven days over all treatment combinations

Table 3.1.6 Spore sizes of *P. clematidina* isolates and herbarium specimens

Isolate	Spore size (*um*) Length	Width
LB	6.6 a	2.6 a c d
MT	3.9 b	1.7 b f
EM	6.9 a c d	2.9 c
HD	6.7 a c	2.8 c
RC	7.0 a c d	2.8 c
A. clematidina 215890	7.6 c d	2.3 a e
A. clematidina 215108	6.9 a c d	2.7 a c d
A. clematidina 2503	7.9 d	2.1 e f
A. indusiata 1198	17.0	4.5
P. herbarum 2431	7.6 c d	2.4 d e
D. vitalbae 620	4.2 b	1.1
P. clematidina 1267	9.0	1.7 b

CV = 8.7% n=20

Values followed by the same letter (in the same column) are equivalent (DNMRT p=0.05)

growth and sporulation were best on PDA. Overall isolate responses to treatment combinations are shown in table 3.1.3.

3.1.4.3 Growth of Type Cultures

Three-way analysis of colony diameter after 7 days indicated significant ($p<0.001$) growth differences between isolates (table 3.1.4). Isolates were ranked on overall mean colony diameter (table 3.1.5).

3.1.5 Herbarium Specimens

New Zealand isolates and 7 '*Phoma*-like' fungi (herbarium specimens) were compared, to indicate possible isolate relationships, by ANOVA analysis of spore length and width (table 3.1.6).

3.1.6 Classification of New Zealand Isolates into Strains

New Zealand isolates were classified into three strains based on isolate associations after Duncan's New Multiple Range Test of colony and growth characteristics, spore size, pathogenicity, toxin production and response to fungicides (see appendix 7). Isolate MT represented one strain, while isolate EM represented an intermediate strain between this strain, and the strain represented isolates by LB, HD and RC.

3.2 DISEASE AND SYMPTOM PROGRESSION

3.2.1 Introduction

"Wilt", the most obvious symptom that develops following fungal colonisation of *Clematis* tissue, is the final symptom of the disease complex. Gloyer (1915a, 1915b) recognised that leaf spotting and internodal stem lesions were also symptoms; plants were killed by pathogen growth from the primary infection site into the stem resulting in "stem girdling".

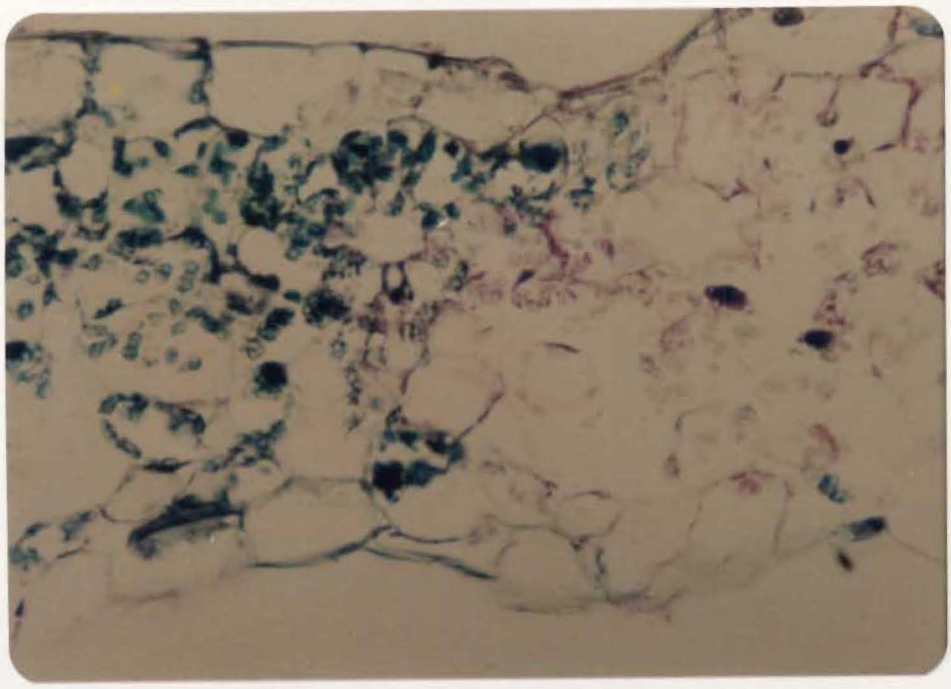
3.2.1 The Leaf Spot

Two zones can be distinguished in leaf spots (figure 3.2.1); a brownish centre surrounded by a black region. This zonation was also observed in artificially produced leaf spots (see figure 3.5.2). Boundaries between the leaf spot zones, and between the black outer zone and surrounding leaf tissue were distinct and sharp. The leaf spot outer edge was sharply defined in transverse sections (TS) stained with Pianeze III_B though the stain reaction varied considerably (figures 3.2.2 and 3.2.3). This was probably due to incorrect differentiation during the staining procedure. Staining with Azur *II* also gave good resolution of the spot edge (figure 3.2.4 & 3.2.5). Hyphae were not present at the leafspot margin, but were observed 4-5 plant cell diameters back from the spot edge (figure 3.2.3). Leaf cell chloroplasts are slightly indistinct as FAA

Figure 3.2.1 Leafspots

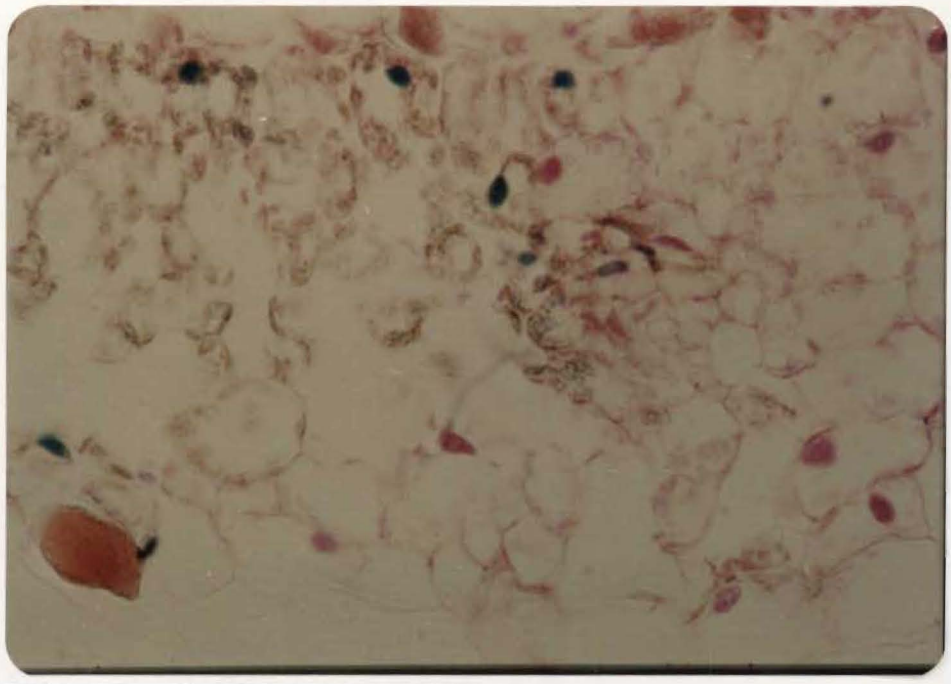
Nine day old leafspots on 'Lady Betty Balfour'

Figure 3.2.2 Margin of leafspot



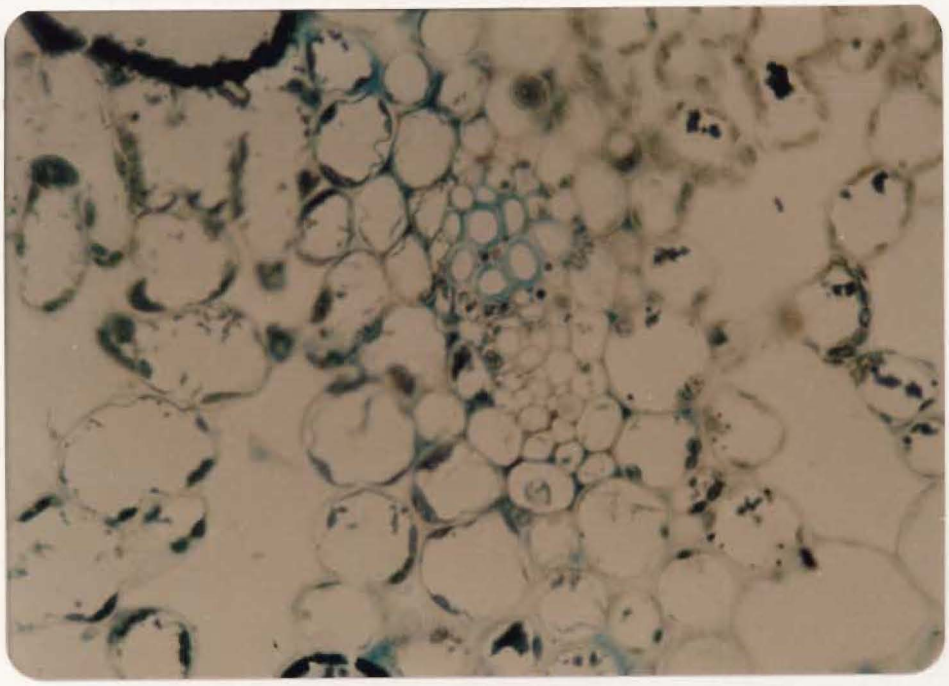
Healthy tissue (pink) to right
(8 *u*m wax/ Pianeze III_B) x400 (TS)

Figure 3.2.3 Edge of leafspot showing ramifying hyphae



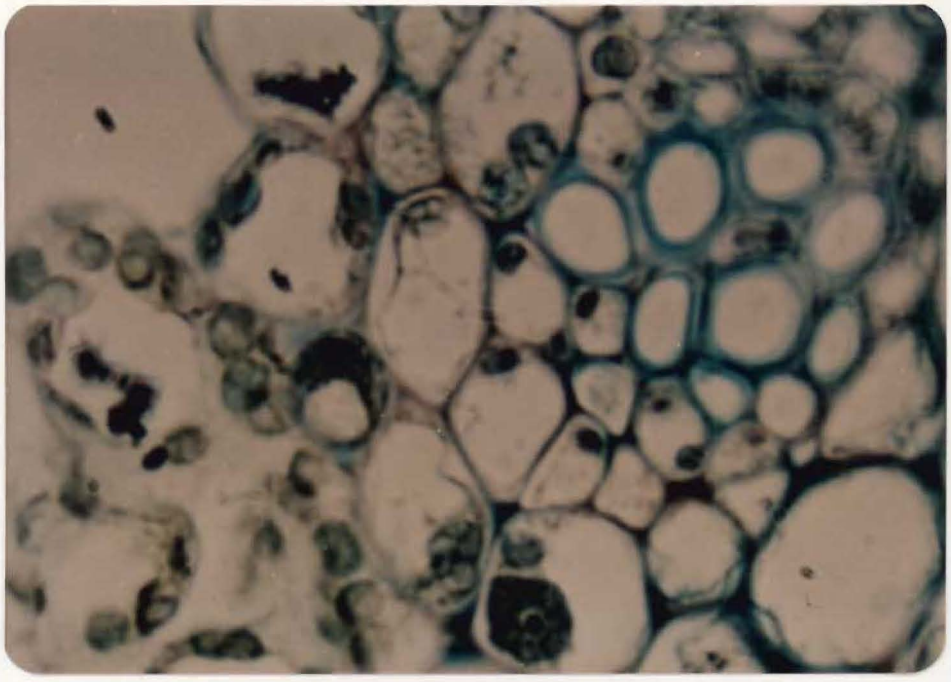
Healthy tissue to right, and hyphal fragments (pink)
in dead tissue to left (8 *u*m wax/ Pianeze III_B) x1000
(TS)

Figure 3.2.4 Cells at leafspot margin



Collapsed cytoplasm on diseased side of leafspot
(4 *u*m Spurrs/ Azur *II*) x400 (TS)

Figure 3.2.5 Cellular detail at leafspot edge



Healthy tissue with normal cytoplasm to right, diseased tissue with collapsed cytoplasm to left
(4 *u*m Spurrs/ Azur *II*) x1000 (TS)

facing page 46

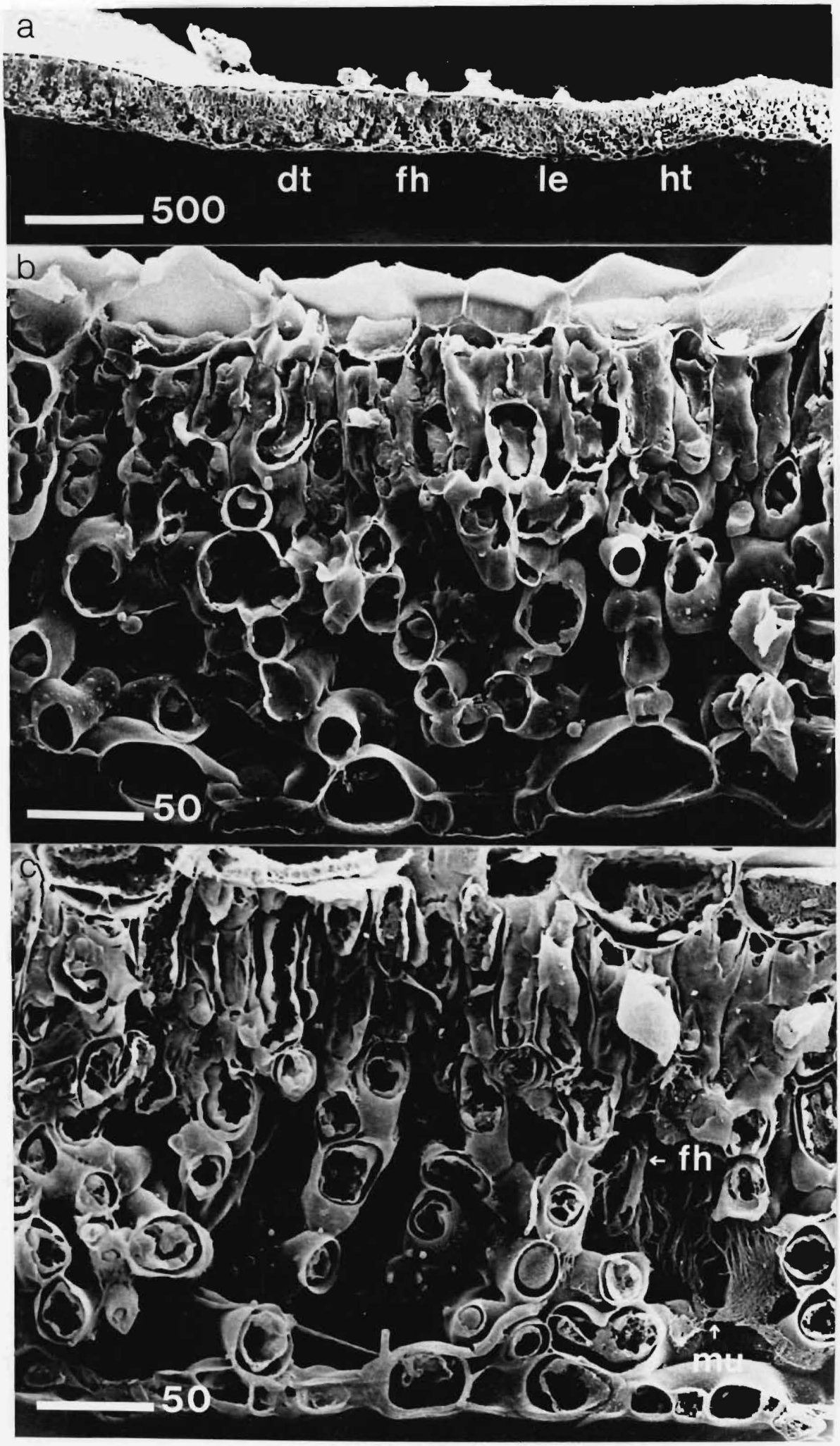
Plate 1 Scanning electron micrographs of leafspot edge (I)

(a) Area of freeze-fractured leaf containing leaspot

(b) Healthy leaf tissue showing 'clean' cell appearance after freeze-fracture

(c) First observed hypal fragments (*c* 0.6mm from spot edge) among leaf cells with collasped cytoplasm. 'Mucilage' material appears in the intercellular spaces

dt	diseased tissue
fh	first hyphae
ht	healthy tissue
le	lesion edge
mu	mucilage


a
dt
fh
le
ht
500
b
50
c
fh
mu
50

facing page 47

Plate 2 Scanning electron micrographs of leafspot edge (II)

(a) Edge of leafspot clearly defined as a sharp boundary (black and white arrows) between 'clean' healthy cells and diseased 'mucilage' cells

(b) Ramifying hyphae in diseased tissue further back from spot margin

dt	diseased tissue
hy	hypha
ht	healthy tissue
le	lesion edge
mu	mucilage

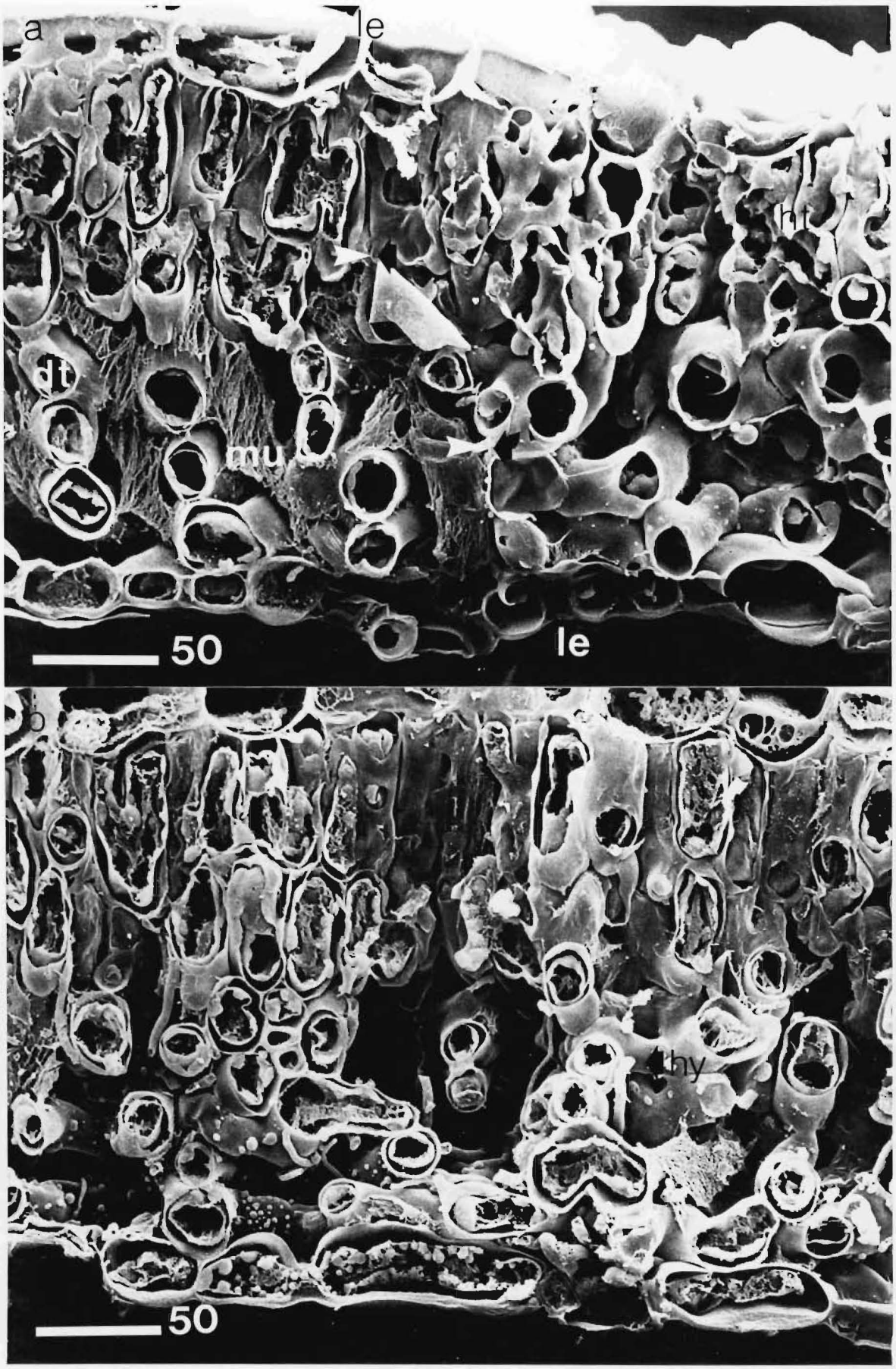
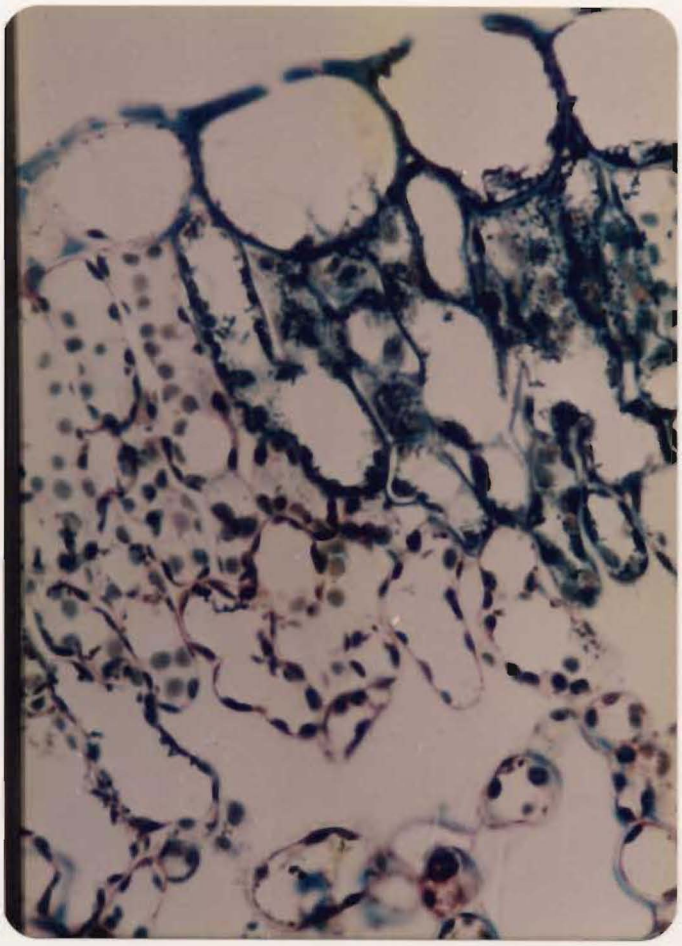

a
le
hy
pt
mu
50
le
b
thy
50

Figure 3.2.6 Leafspot margin



Diseased tissue to right with collapsed cytoplasm
(4 *u*m Spurrs/ Pianeze III_B) x400 (TS)

facing page 49

Plate 3 Choroplasts from neighbouring cells across leafspot margin

(a) Chloroplast and mitochondria from healthy cell showing clearly resolved internal organisation and limiting membranes

(b) Choroplast on edge of spot with indistinct grana beginning to separate, and granular ground plasm

(c) Choroplast showing thylakoid and ground plasm disintegration. Mitochondrial internal organisation still evident although envelope indistinct

(d) Limiting membranes of chloroplast and mitochondria are resolvable

(e) Complete amorphising of chloroplast contents and limiting membrane undistinguishable. Mitochondrial cristae appear to have lost intergity

ch	cloroplast
cw	plant cell wall
pl	plasmalemma
mt	mitochondria
st	starch grain
tn	tonoplast
va	vacuole

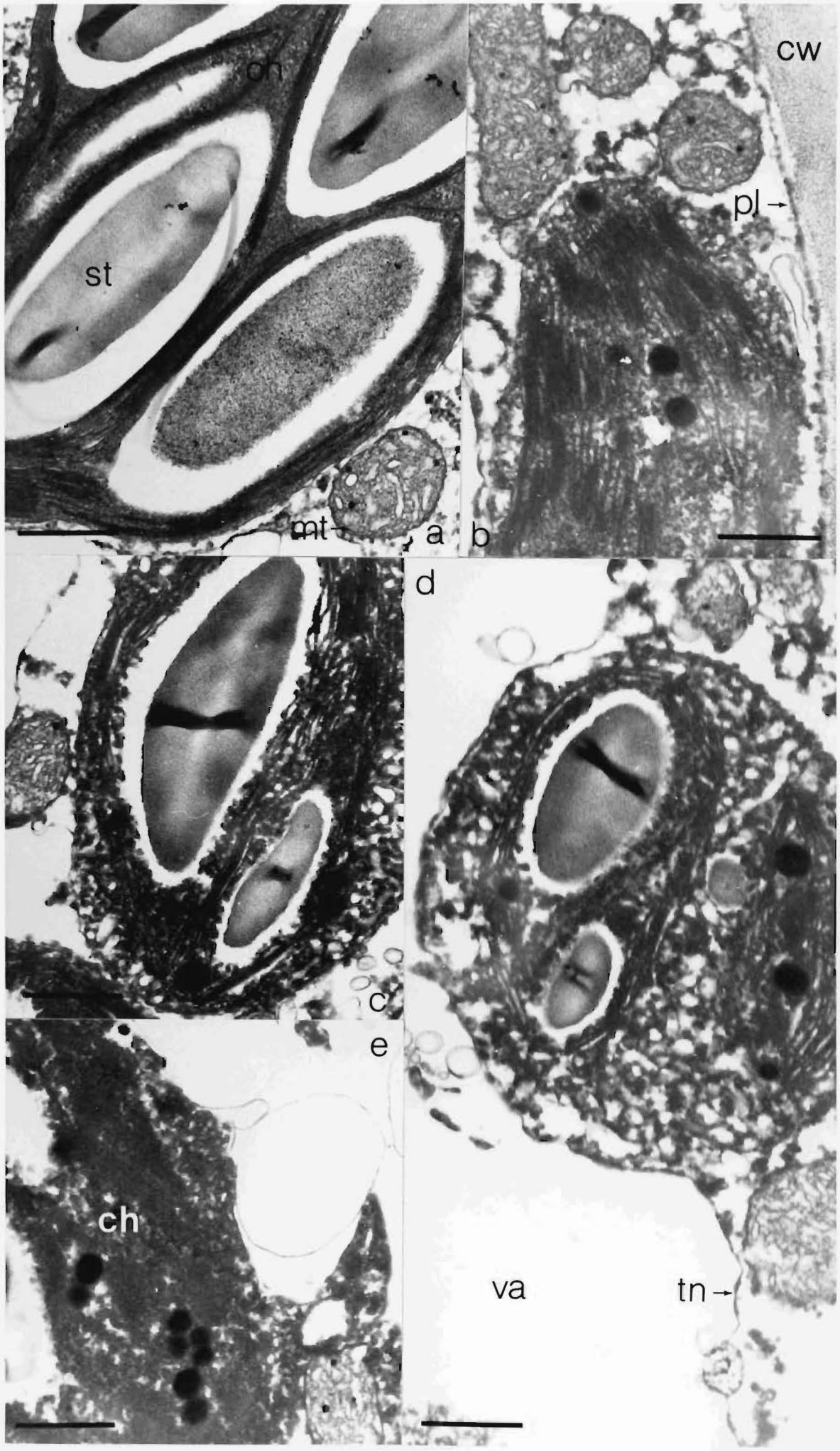

ch
st
mt
a
cw
pl
b
c
d
e
ch
va
tn

Figure 3.2.7 Leafspot expansion

Leafspot expansion in leaf laminae

Figure 3.2.8 Lesion development into petiole

Leafspot lesion expanding into petiole of 'Lady Betty Balfour', a wilt susceptible cultivar

Figure 3.2.9 "Leaf wilt"

Petiole lesions developed in both directions often causing the leaf to wilt before the plant

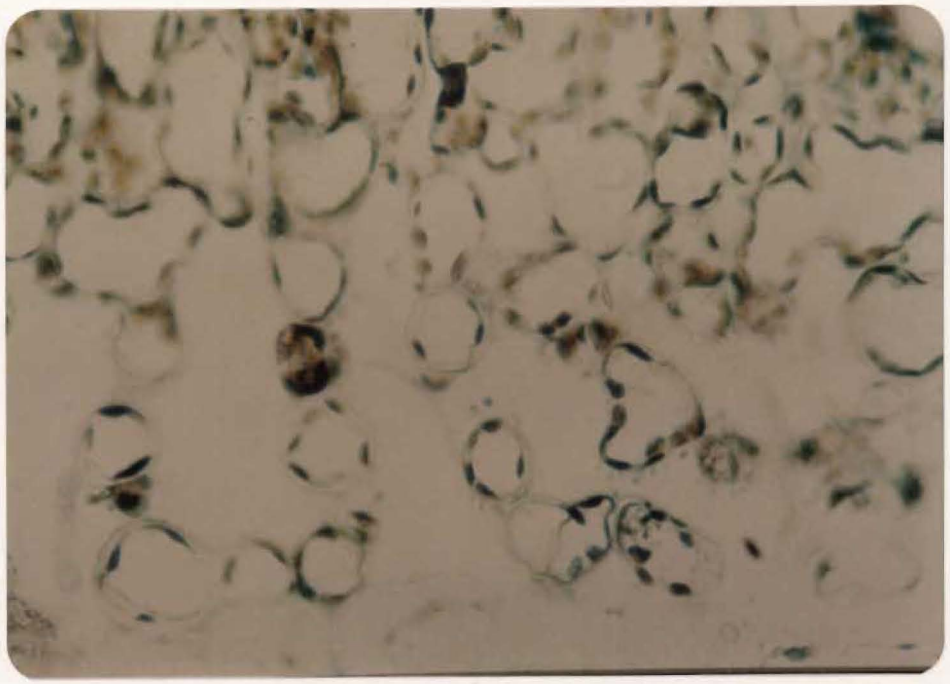
Figure 3.2.10 Fungal spread into node

The leaf laminae and petiole are dead. Hyphae are penetrating the node, eventually resulting in wilt

Figure 3.2.11 Wilted *Clematis* plant

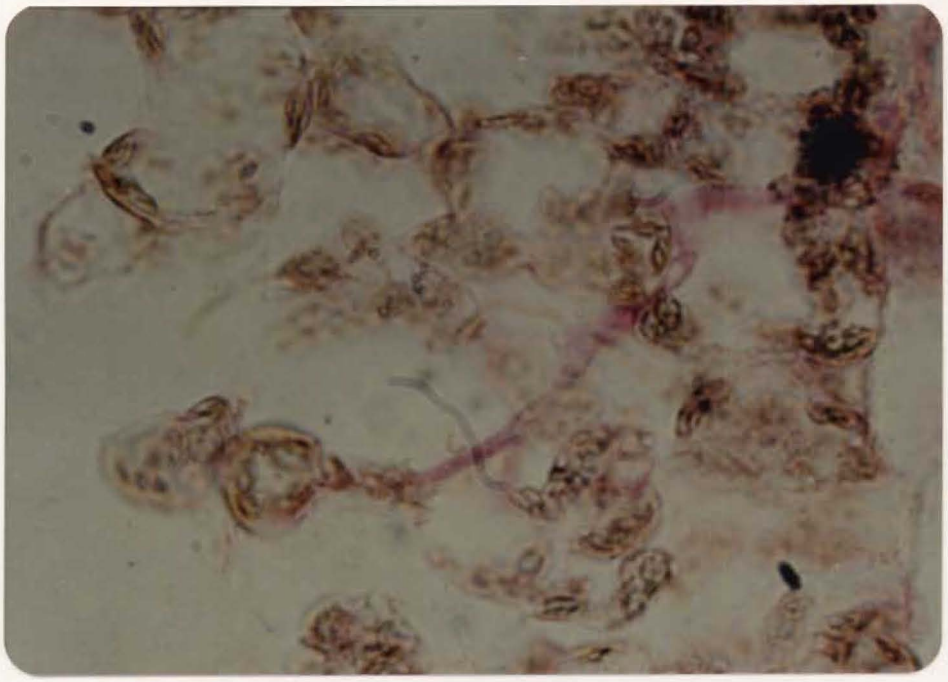
Typical wilt symptom where plant has wilted and died above infected node while tissue below node is alive. New growth apparent from node immediately beneath point of wilt (scale bars = 1cm)

Figure 3.2.12 Ramifying hyphae in leaf lesion



Hyphae ramifying through diseased tissue
(4 *u*m Spurrs/ Pianeze III_B) x400 (TS)

Figure 3.2.13 Hyphae ramifying through dead leaf tissue



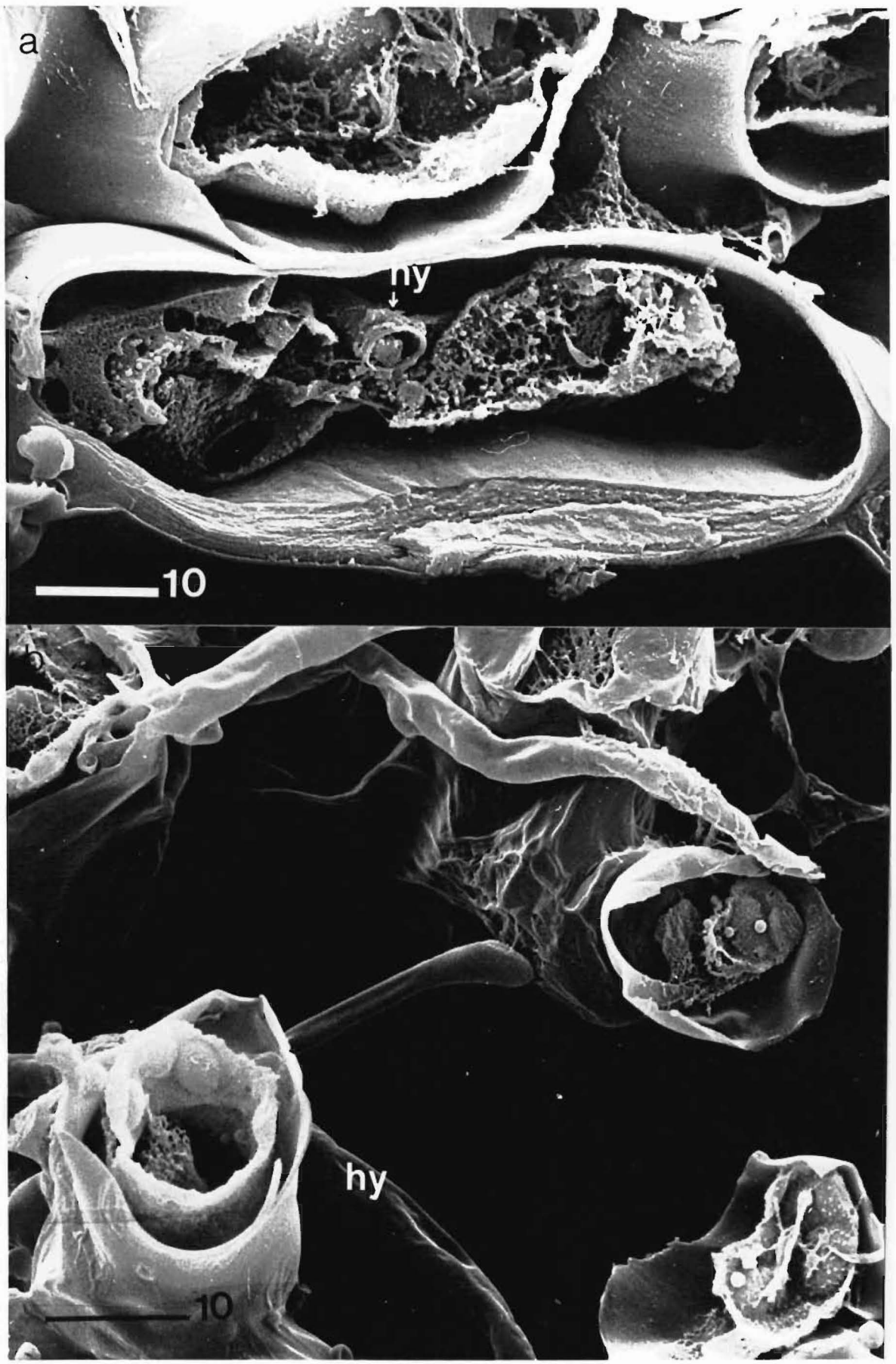
Hyphae (pink) ramifying through dead mesophyll and
palisade cells (8 *u*m wax/ Pianeze III_B) x1000 (TS)

facing page 55

Plate 4 Scanning electron micrographs of ramifying hyphae

(a) Intracellular hyphae in freeze-fractured leaf lower epidermal cell. Hyphae does not appear to have penetrated into cytoplasm remains
(b) Intracellular hyphae ramifying between and through mesophyll leaf cells

hy hypha


a
hy
10
b
hy
10

facing page 56

Plate 5 Transmission electron micrographs of ramifying hyphae

(a) Double nucleated hyphal tip (LS) between two plant cell walls
(b) Plant cell wall appears to be degraded in front of the hyphal tip suggesting enzymatic activity
(c) Hypha (TS) appears attached to the inside of the plant cell wall. Hypha does not appear to have penetrated cytoplasm remains (c.f. plate 4 a)

at	attachment point
cr	cytoplasm remains
cw	plant cell wall
nu	nucleus

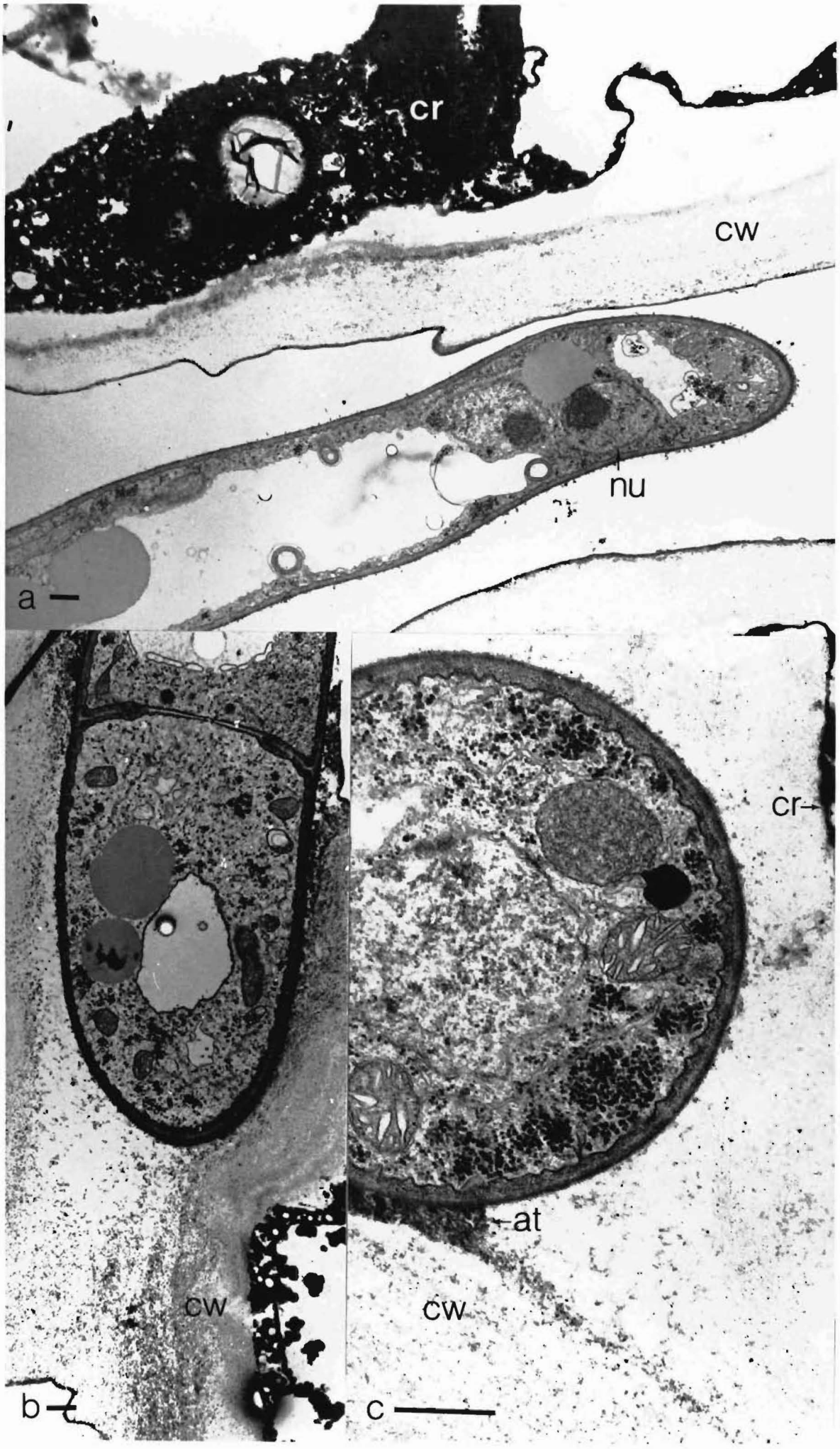

cr
cw
nu
a
b
cw
cr
at
cw
c

Figure 3.2.14 Exterior of rotted node

Grey-black discolouration of rotted node. Pycnidia are visible above, below and on the petiole of the node (x5)

Figure 3.2.15 Interior of rotted node

Stem split at rotted node (LS) . Tissue appears healthy below the rot. All tissues of the node are affected by the rot (x5)

Figure 3.2.16 Stem rot margin

A sharp boundary is apparent between rotted and healthy tissue in the stem (x10) (LS)

fixes material by agglutination, rather than cross-linking (i.e. glutaraldehyde), giving a 'softer' image. The sharply defined leafspot edge was also evident in scanning electron micrographs of freeze-factured leafspots (plates 1 and 2). The first hyphae were observed *c* 0.6mm from the spot edge (plate 2).

Glutaraldehyde fixed tissue best demonstrates cytoplasm differences between cells on either side of the leaf spot boundary. Cytoplasm of cells on the 'diseased side' is contracted and individual organelles are indistinguishable, in contrast to cell cytoplasm on the 'healthy side' (figure 3.2.6). Limiting membranes of cell organelles are indistinct and ground plasm very granular. Transmission electron micrographs of leaf cells of the spot margin show deterioration of chloroplast and mitochondria limiting membranes and internal structure (plate 3). There was a reduction in thylakoid number and granal size in 'healthy' tissue, probably due to the low light intensity of the growth room.

3.2.3 Disease Progression

In the glasshouse leaf spots expand (figure 3.2.7) in the leaf laminae; the lesion then proceeds down the petiole (figure 3.2.8). If the petiole was the site of infection, the lesion expands in both directions girdling the petiole causing "leaf wilt" (figure 3.2.9). Eventually the leaf and petiole are killed (figure 3.2.10), although time taken until the plant wilts is extremely variable (see section 3.6).

Extensive ramification of inter- and intra-cellular hyphae through leaf cells occurs (figures 3.2.12 and 3.2.13, plates 4 and 5). The symptom of rotted stem tissue is almost universal (figures 3.2.14 and 3.2.15). Stem tissue below the node is still green and alive with a sharp boundary apparent between dead and healthy tissues (figure 3.2.16).

3.3 CONIDIOGENESIS AND SPORE RELEASE

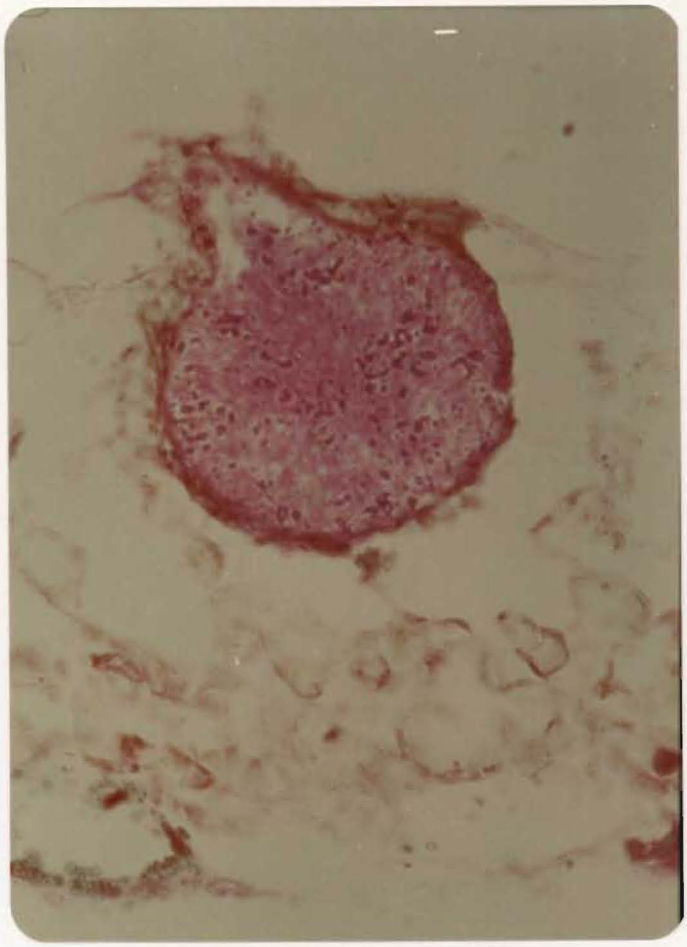
3.3.1 Pycnidia and Sporulation

Mature pycnidia were apparent on infected leaf discs *c* 4 days after inoculation. Pycnidia, globose and embedded in leaf tissue remains (figure 3.3.1, plate 6), consist of a wall composed of 1-4 cells (plate 7) enclosing the conidiophores (plate 8). A collarette surrounds the conidiogenetic locus (plate 9) of the conidiogenous cells, indicating phialidic conidial ontogeny (*sensu* Boerema and Bollen 1975). Spores were hyaline, predominately one-celled and measured 3.9-7.0 by 1.7-2.9 *u*m.

3.3.2 Spore Release

Pale saffron-salmon (Rayner 1970) cirrhi are extruded by mature pycnidia incubated in a moisture chamber overnight (figure 3.3.3). high humidity during growth room experiments induced spore release (figures 3.3.4 and 3.3.5). Eventually masses of extruded spores cover the abaxial leaf surface (plates 10 and 11).

Figure 3.3.1 Pycnidium of *Phoma clematidina*



Globose pycnidium erumpent onto abaxial leaf surface (8 *u*m wax/ Pianeze III_B) x400 (TS)

facing page 62

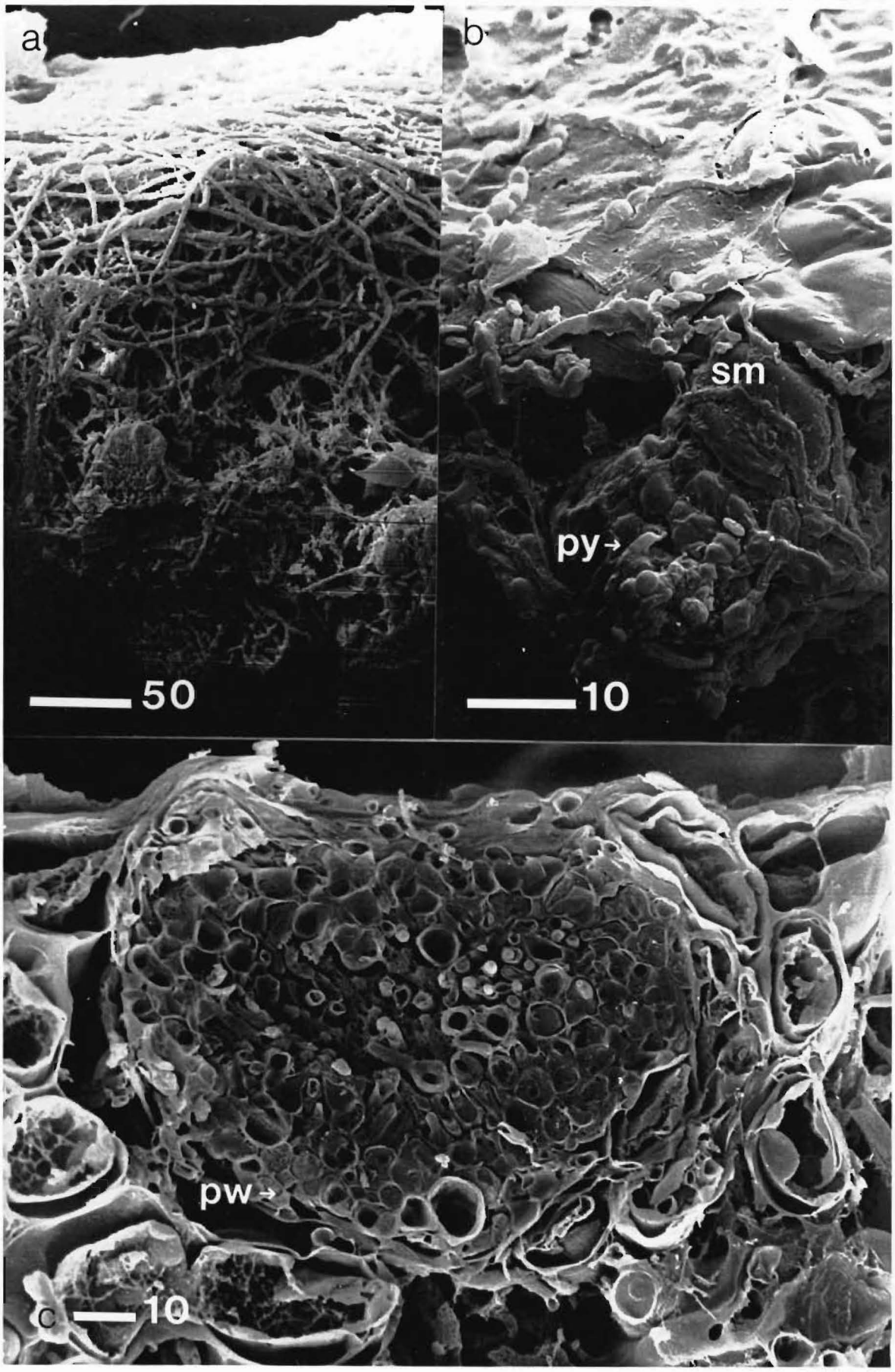
Plate 6 Pycnidial formation and structure

(a) Extensive hyphal ramification through and over leaf tissue prior to formation of pycnidia

(b) Pycnidia formation. Fracture plane has gone round outside of young pycnidium forming near leaf abaxial surface

(c) Mature sporulating pycnidium. Fracture plane is not medial but is near the back wall. Conidiogenetic cells and the pycnidial cell wall are clearly visible

pw	pycnidial wall
py	pycnidium
sm	stoma

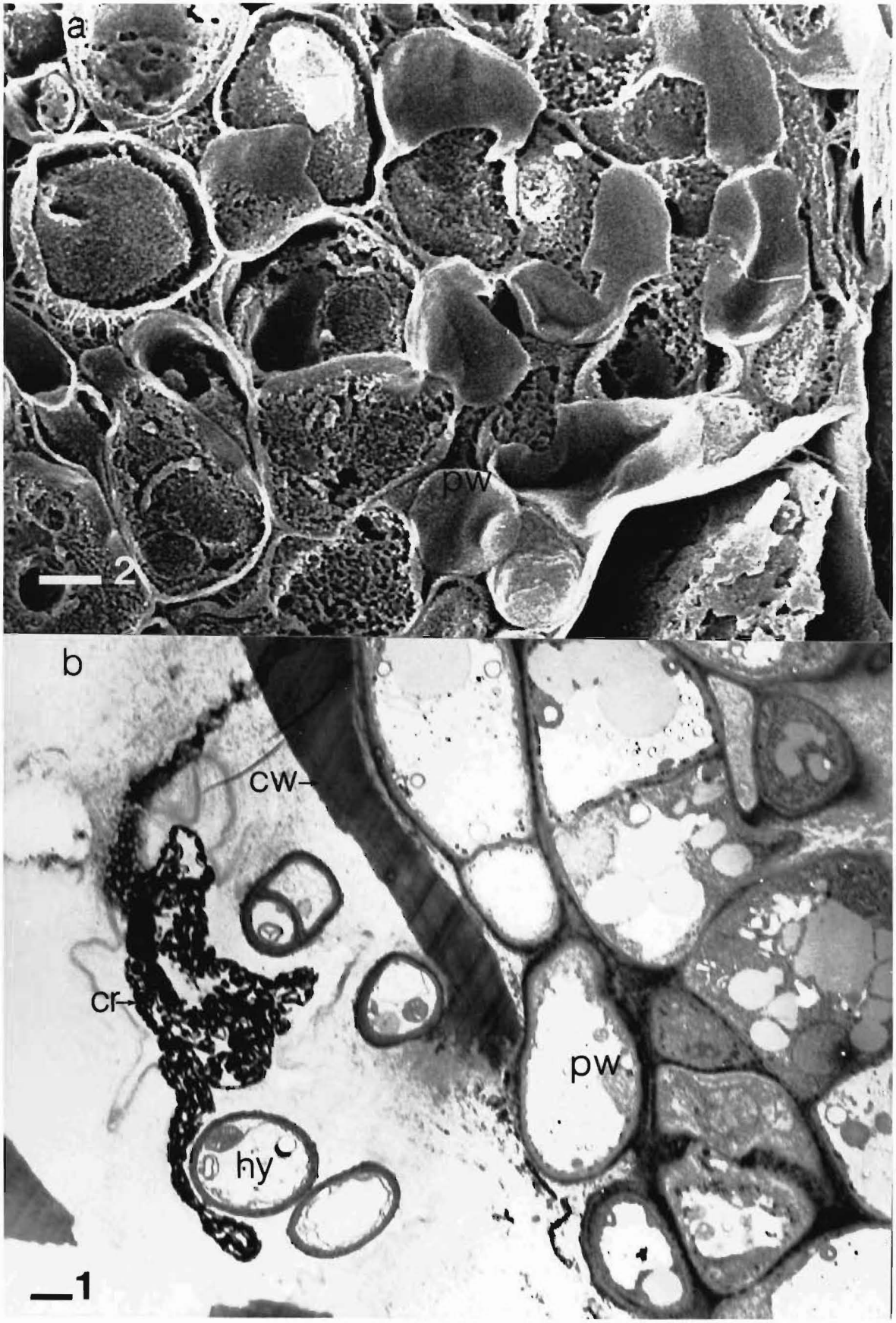

a
50
b
sm
py→
10
pw→
c
10

facing page 63

Plate 7 Pycnidial wall

(a) The pycnidial wall (2-3 cells thick) appears to be formed exclusively of fungal material. Conidiogenetic cells are visible inside the wall
(b) Thickness of the pycnidial wall is variable, ranging from one to four cells. Plant cell wall and cytoplasm remains, and hyphae are evident. A conidiogenetic cell is sporulating into the pycnidial matrix

cr	cytoplasm remains
cw	plant cell wall
hy	hyphae
pw	pycnidial cell wall

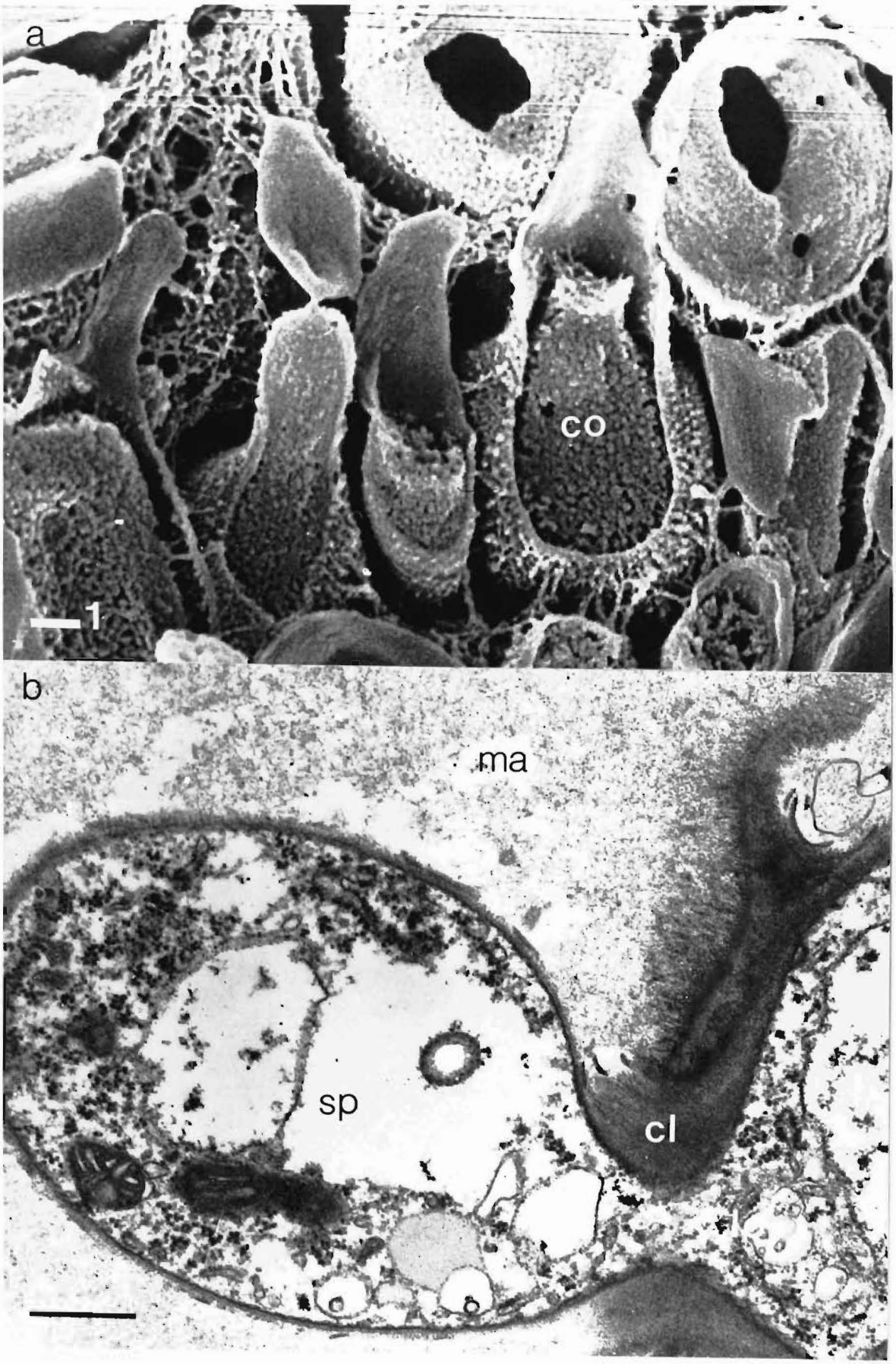

a
pw
2
b
cw
cr
pw
hy
1

facing page 64

Plate 8 Conidiophores and conidiogenesis

(a) Freeze fracture of conidiogenetic cells with forming spores attached
(b) Conidiogenesis. A collarette of the remains of spore walls of preceeding spores surrounds the conidiogenetic locus

cl	collarette
co	conidiogenetic cell
lo	conidiogenetic locus
ma	matrix
sp	spore


a
co
1
b
ma
sp
cl

facing page 65

Plate 9 Phialidic conidiogenesis

(a) Conidiogenetic cell appears to be budding two spores (arrowed)

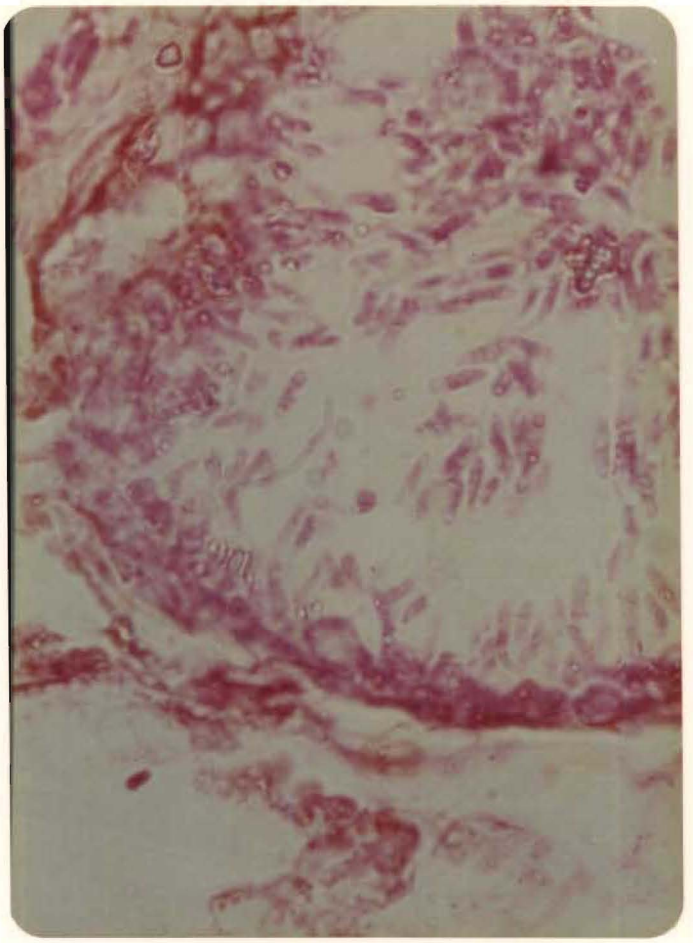
(b) Mature spore about to secede into matrix

(c) Collarette surrounding the conidiogenetic locus of the conidiophore. Matrix which fills the pycnidium is evident in this plate

cl collarette

co conidiogenetic cell

Figure 3.3.2 Conidia within pycnidium

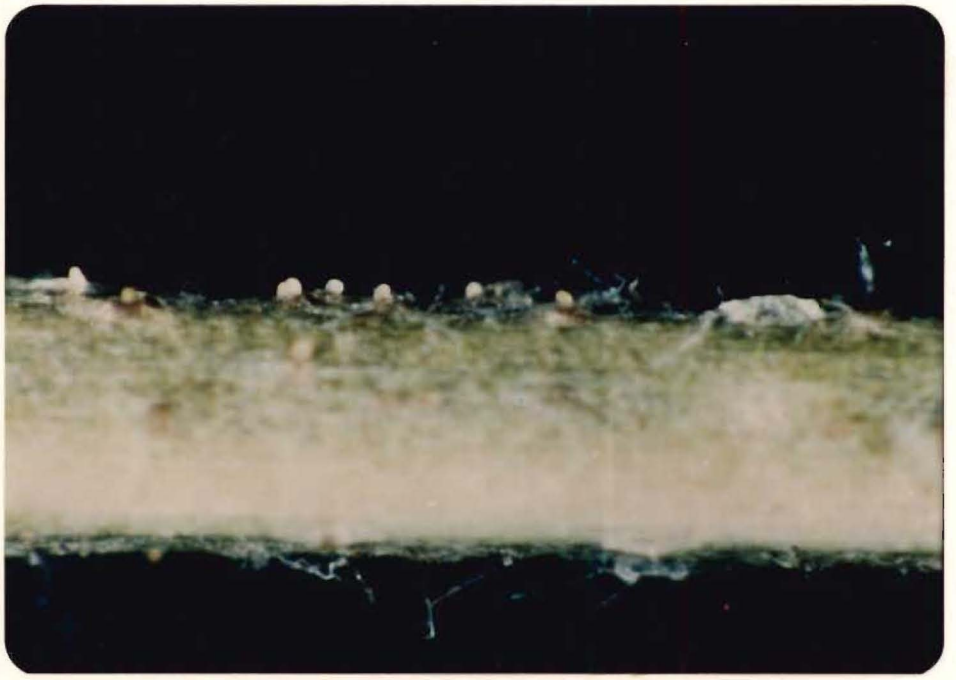


Conidiogenetic cells and pycnidial wall details are not distinguishable with the light microscope
(8 *u*m wax/ Pianeze III_B) x1000 (TS)

Figure 3.3.3 Spore cirrhi on leaf surface

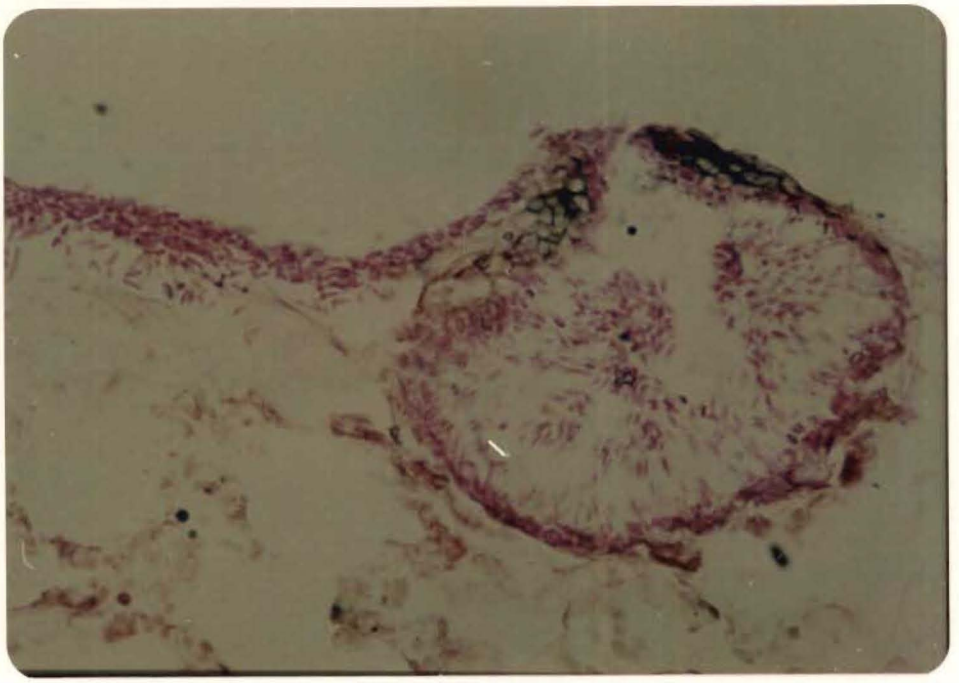
Cirrhi extruding from pycnidia embedded in leaf (x10)

Figure 3.3.4 Spore cirrhi on *Clematis* stem

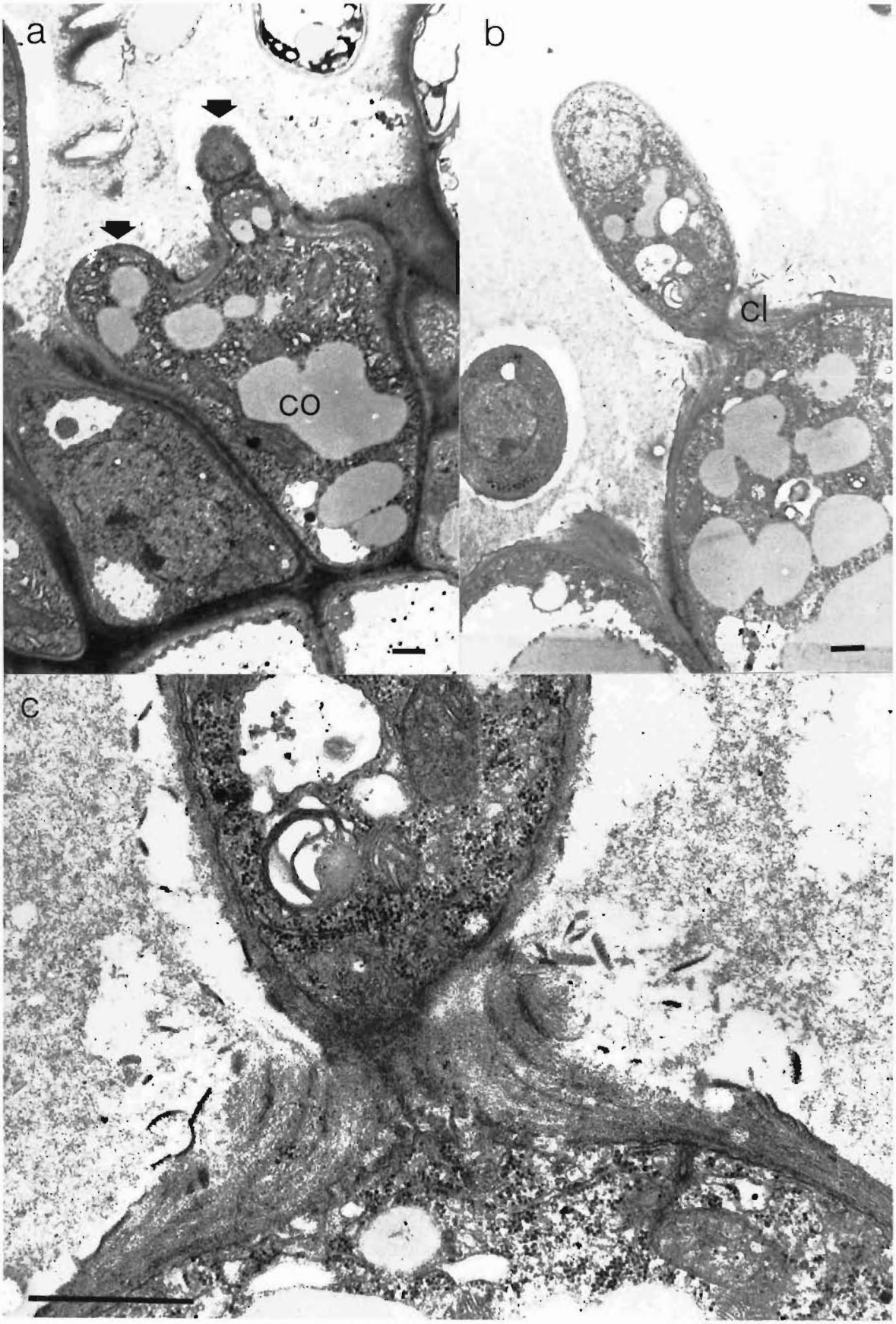


Spore cirrhi extruding from *Phoma clematidina* pycnidia embedded in stem (x10)

Figure 3.3.5 Spore dehiscence from pycnidium



Spores extruded as a cirrhus onto abaxial leaf surface (8 *u*m wax/ Pianeze III_B) x400 (TS)

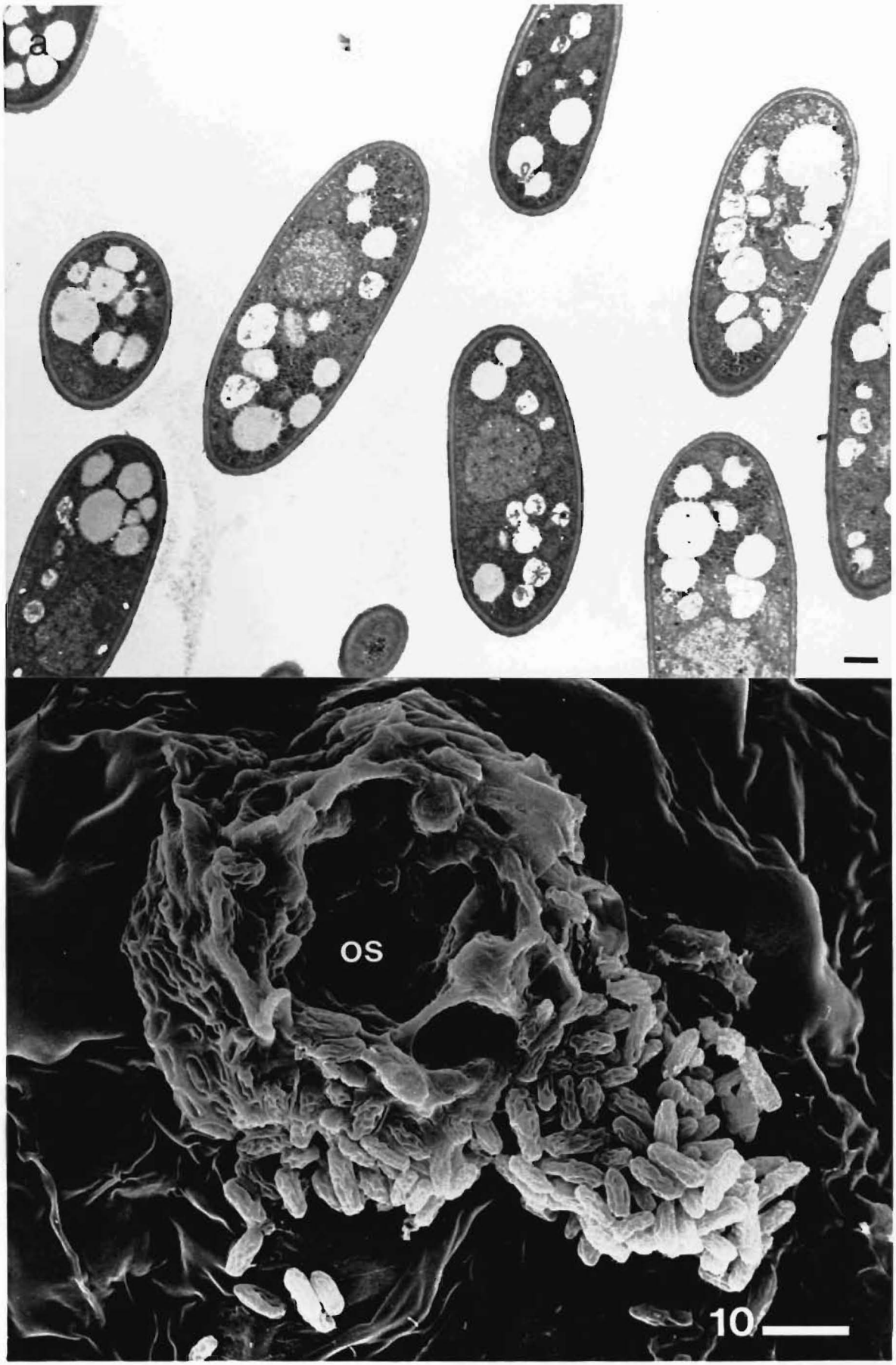

a
co
b
cl
c

facing page 69

Plate 10 Conidia and conidial dehiscense

(a) Typical one-celled spores in pyncidial matrix. The light areas are probably oil-filled vesicles (LS and TS).
(b) Vertical view of pycnidial ostiole with spores beginning to dehisce down side

os ostiole

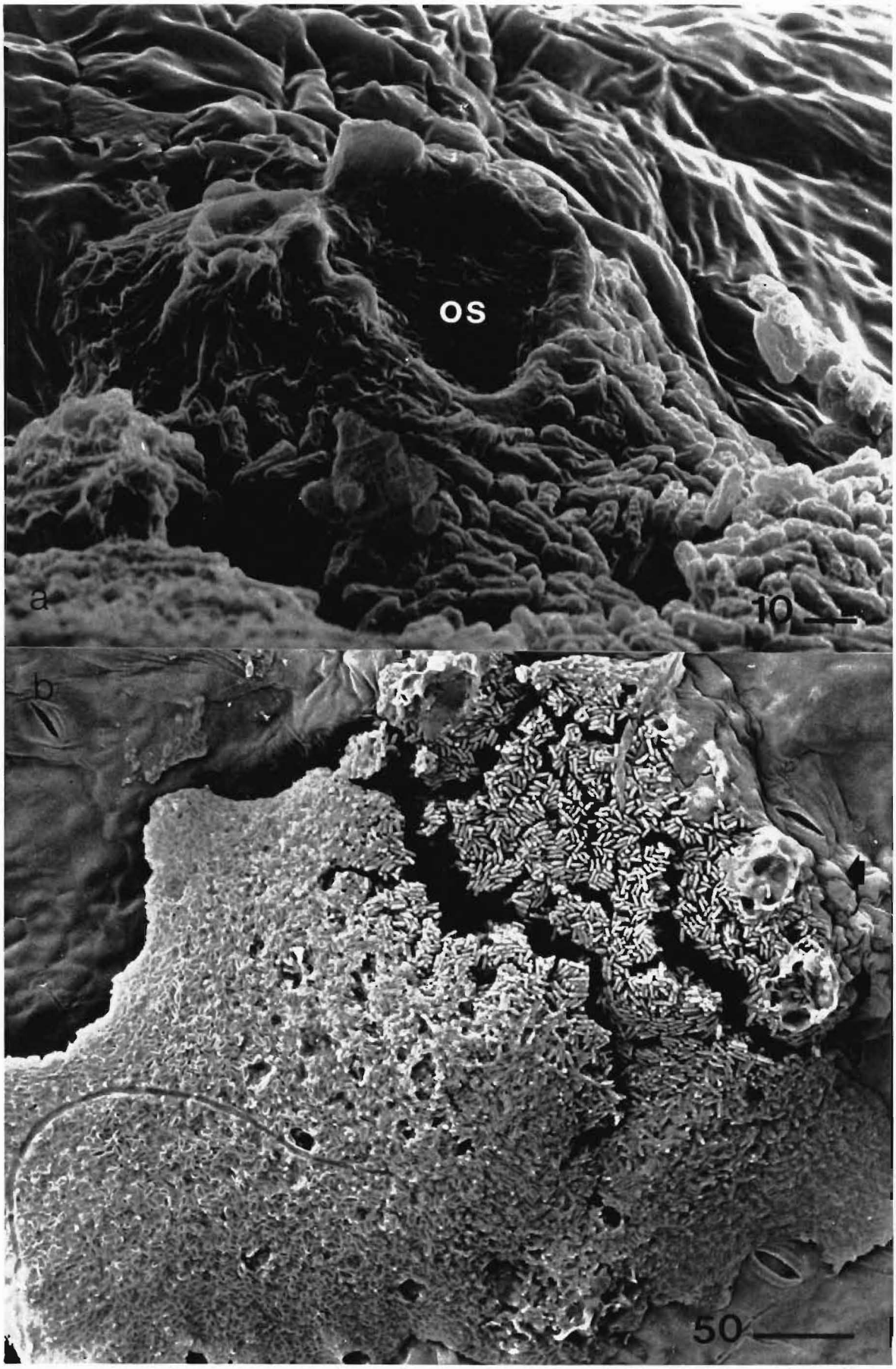

a
OS
10

facing page 70

Plate 11 Spore dehiscence

(a) Pycnidial ositole with spores in matrix in foreground
(b) Two pycnidia (arrowed) surrounded by masses of spores

os ostiole


OS
a
10
b
50

3.4 SPORE GERMINATION

3.4.1 Choice of Spore Preparation Method and Concentration

Spore suspensions of $1x10^6$ conidia/ml produced by method A were used for further investigations. Method A produced suspensions in which spore germination was consistent up to conidial concentrations of $1x10^6$ conidia/ml (table 3.4.1), while germination of spores in $1x10^6$ conidia/ml suspensions produced by method B was significantly ($p<0.001$) less at this concentration.

The difference in germination of spores in suspensions produced by the two methods was probably due to carry over of water soluble nutrients from agar when the suspension was made. Inclusion of matrix material in the suspension may have also enhanced germination as the matrix is reported to promote germination (Chung and Wilcoxon 1969), although inhibition is also noted (Louis and Cooke 1985). Variation of germination in spore suspensions prepared by the two methods was equivalent (Barletts test), though McCallan and Wilcoxon (1939) had found that a 15 to 20 fold decrease in germination variation could be effected by centrifuging, decanting and resuspending spores in distilled water.

3.4.2 Germination of Isolate Spore Suspensions

Germination of T5 spore suspensions of four New Zealand *P. clematidina* isolates is recorded in table 3.4.2. ANOVA analysis of mean spore germination at $1x10^6$ conidia/ml concentration indicated no differences between isolates.

3.4.3 Growth Room Germination

ANOVA analysis indicated no difference in spore germination between suspensions germinated in the growth room or an incubator.

3.4.4 Leaf Disc Germination

Germination was evident after 24h incubation of spore suspensions on the abaxial surface of non-wounded leaf discs (plate 12).

3.5 LEAF DISC INFECTION

3.5.1 Floating Solutions

No visual differences were observed between leaf discs floated on distilled water, or kinetin solution for 7 days (figure 3.5.1).

3.5.2 Infection Studies

Aggressiveness (the number of days for a visible lesion to develop beyond the point of inoculation) was recorded for each isolate against the cultivar from which it

Table 3.4.1 Effect of preparation method and spore concentration on conidial germination

Preparation method	Spore concentration (conidia/ml) $1x10^5$	$5x10^5$	$1x10^6$	$5x10^6$	$1x10^7$
	percent germination				
A (unwashed)	98.4	97.9	97.5	66.5	32.2
DNMRT p=0.05					
B (washed)	96.5	85.7	57.0	39.6	33.9
DNMRT p=0.05					
CV = 9.9%				n=5	

Table 3.4.2 Effect of spore concentration on germination of four New Zealand isolates

Isolate	Spore concentration (conidia/ml) $1x10^5$	$1x10^6$	$1x10^7$
	percent germination		
MT	98.6	96.6	4.6
EM	98.6	98.6	34.0
HD	98.6	98.0	31.2
RC	97.6	96.0	4.8
CV = 1.69%		n=5	

facing page 73

Plate 12 Spore germination

(a) Germinated spore after 24h on abaxial surface of leaf. Germ tube appears to be growing around the stomata
(b) Germination of $1x10^6$ conidia/ml on unwounded abaxial leaf surface of 'Lady Betty Balfour' after 24h incubation.
(c) Conidial germination on leaf vein.

gt	germ tube
sm	stoma
sp	spore

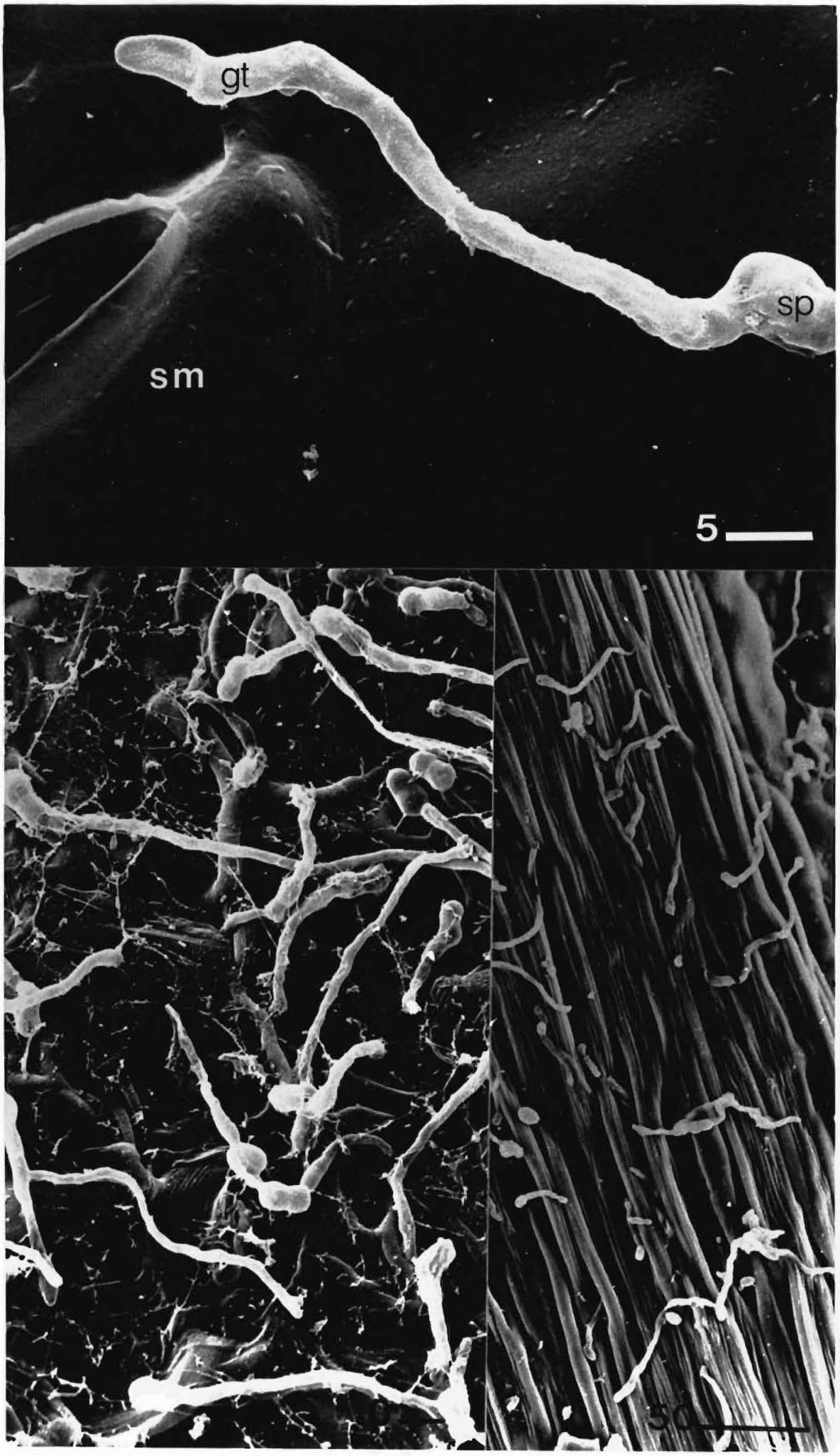
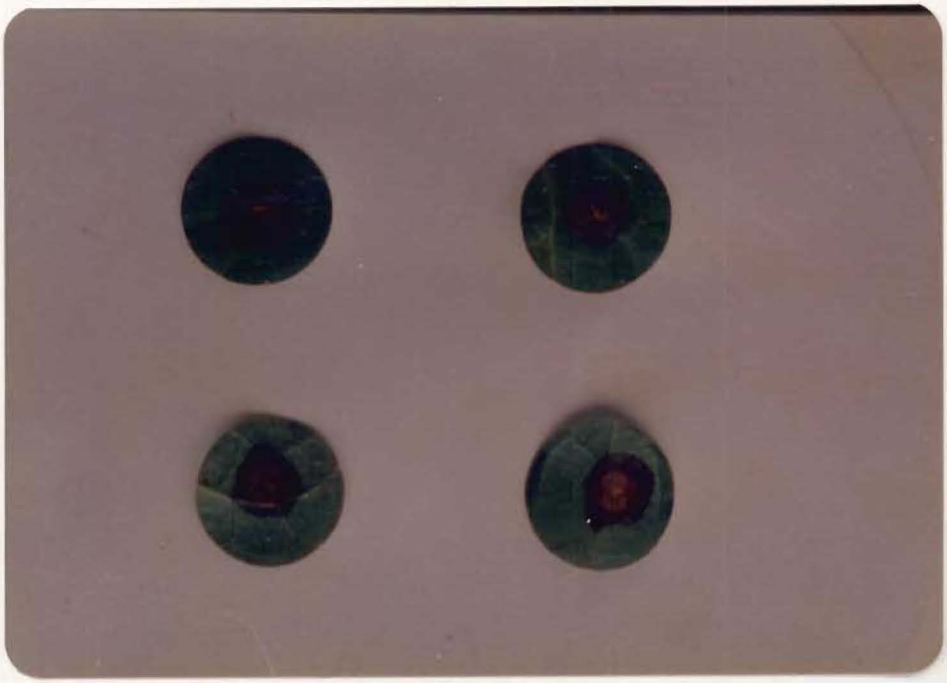

gt
sp
sm
5
5

Figure 3.5.1 Appearance of leaf discs after 7 days

A B A B

Adaxial and abaxial surfaces of discs floated on 50 *u*g/ml kinetin solution (A) or distilled water (B)

Figure 3.5.2 Leaf spots produced *in vitro*



Seven day leafspots on 'Lady Betty Balfour' leaf discs

Table 3.5.1 Effect of leaf disc treatment on production of lesions: 'Lady Betty Balfour' discs inoculated with isolate LB spore suspension

Day	Leaf disc treatment		
	Non-wounded	Point wounded	Scrape wounded
percent leaf discs with visible lesion beyond inoculation point			
2	0	0	0
4	12	8	96
7	20	56	96

(1) control leaf discs inoculated with T5 solution did not develop lesions n= 25

(2) chi-square test

day	ns
treatment	p<0.001
day x treatment	p<0.05

Table 3.5.2 Effect of leaf disc treatment on production of lesions: 'Montana' discs inoculated with isolate MT spore suspension

Day	Leaf disc treatment		
	Non-wounded	Point wounded	Scrape wounded
percent leaf discs with visible lesion beyond inoculation point			
2	0	4	4
4	16	20	40
7	20	24	40

(1) control leaf discs inoculated with T5 solution did not develop lesions n=25

(2) chi-square test

day	ns
treatment	p<0.001
day x treatment	ns

Table 3.5.3 Effect of leaf disc treatment on production of lesions: 'Ernest Markham' discs inoculated with isolate EM spore suspension

Day	Leaf disc treatment: Non-wounded	Point wounded	Scrape wounded
percent leaf discs with visible lesion beyond inoculation point			
2	8	4	36
4	40	64	96
7	44	64	96

(1) control leaf discs inoculated with T5 solution did not develop lesions n=25

(2) chi-square test

day	p<0.001
treatment	p<0.001
day x treatment	ns

Table 3.5.4 Effect of leaf disc treatment on production of lesions: 'Huldine' discs inoculated with isolate HD spore suspension

Day	Leaf disc treatment: Non-wounded	Point wounded	Scrape wounded
percent leaf discs with visible lesion beyond inoculation point			
2	0	0	0
4	0	0	72
7	0	16	96

(1) control leaf discs inoculated with T5 solution did not develop lesions n=25

(2) chi-square test

day	p<0.001
treatment	p<0.001
day x treatment	p<0.01

Table 3.5.5 Effect of leaf disc treatment on production of lesions: 'Rouge Cardinal' discs inoculated with isolate RC spore suspension

Day	Leaf disc treatment: Non-wounded	Point wounded	Scrape wounded
percent leaf discs with visible lesion beyond inoculation point			
2	4	4	44
4	8	64	96
7	16	68	96

(1) control leaf discs inoculated with T5 solution did not develop lesions n=25

(2) chi-square test

day	p<0.001
treatment	p<0.001
day x treatment	ns

Table 3.5.6 Effect of leaf disc treatment on diameter of lesion: 'Lady Betty Balfour' discs inoculated with isolate LB spore suspension

	Leaf disc treatment: Non-wounded	Point wounded	Scrape wounded
	average diameter of lesions (mm)		
Day 4	1.3	2.5	4.2
DNMRT p=0.05	_____	_____	
Day 7	6.2	5.7	10.1
DNMRT p=0.05	_____	_____	

n=25

Table 3.5.7 Effect of leaf disc treatment on diameter of lesion: 'Montana' discs inoculated with isolate MT spore suspension

	Leaf disc treatment		
	Non-wounded	Point wounded	Scrape wounded
	average diameter of lesions (mm)		
	1.4	2.7	3.2
DNMRT p=0.05			

n = 25

Table 3.5.8 Effect of leaf disc treatment on diameter of lesion: 'Ernest Markham' discs inoculated with isolate EM spore suspension

	Leaf disc treatment		
	Non-wounded	Point wounded	Scrape wounded
	average diameter of lesions (mm)		
	10.4	10.3	11.7
DNMRT p=0.05	a		a

n = 25

Table 3.5.9 Effect of leaf disc treatment on diameter of lesion: 'Huldine' discs inoculated with isolate HT spore suspension

	Leaf disc treatment		
	Non-wounded	Point wounded	Scrape wounded
	average diameter of lesions (mm)		
	*	4.5	7.9
DNMRT p=0.05			

(1) * no lesions

n = 25

Table 3.5.10 Effect of leaf disc treatment on diameter of lesion: 'Rouge Cardinal' discs inoculated with isolate RC spore suspension

	Leaf disc treatment		
	Non-wounded	Point wounded	Scrape wounded
	average diameter of lesions (mm)		
	4.5	7.5	10.4
DNMRT p=0.05			

n = 25

Table 3.5.11 Production of lesions on inoculated cultivar leaf discs

Isolate	Cultivar(1) Balfour	Montana	Markham	Huldine	Cardinal
	percent leaf discs with visible lesion beyond inoculation point				
LB	100	72	100	100	100
MT	36	36	32	48	52
EM	80	72	100	92	76
HD	100	48	100	100	96
RC	96	48	84	92	88

n=25

(1) 'Lady Betty Balfour' 'Montana' 'Ernest Markham' 'Huldine' 'Rouge Cardinal'

(2) chi-square test

cultivars	$p<0.05$
isolates	$p<0.001$
cultivars x isolates	ns

Table 3.5.12 Lesion size on inoculated cultivar leaf discs

Isolate	Cultivar(1) Balfour	Montana	Markham	Huldine	Cardinal
		average diameter of lesions (mm)			
LB	11.9	3.9	9.4	8.6	7.6
MT	2.2	2.2	2.1	4.7	2.7
EM	6.8	6.7	7.8	6.3	5.0
HD	13.2	5.7	12.4	14.0	9.9
RC	8.6	3.8	8.3	9.9	8.7

n=25

CV = 22.2%

(1) 'Lady Betty Balfour' 'Montana' 'Ernest Markham' 'Huldine' 'Rouge Cardinal'

Table 3.5.13 Overall lesion size on each cultivar caused by all isolates after seven days

	Cultivar				
	Balfour	Montana	Markham	Huldine	Cardinal
	average diameter of lesions (mm)				
Lesion diameter	8.5	4.5	8.0	8.6	6.7
DNMRT					
p=0.05	a b		a	b	
p=0.01	a		a	a	

Table 3.5.14 Overall size of isolate lesions on all cultivars after seven days

	Isolate				
	LB	MT	EM	HD	RC
	average diameter of lesions (mm)				
Lesion diameter	8.3	2.8	6.5	11.0	7.9
DNMRT					
p=0.05	a				a
p=0.01	a				a

Table 3.5.15 Effect of wounding sepal discs on number of lesions: 'Lady Betty Balfour' discs inoculated with isolate LB spore suspension

Sepal disc treatment	Day 2	4
	percent discs with visible lesion beyond inoculation point	
Non-wounded	52	92
Scrape wounded	68	96
Controls[1]		
Non-wounded	4	20
Scrape wounded	0	12

(1) inoculated with T5 solution n=25

(2) chi-square test

day	ns
treatment	ns
day x treatment	ns

was originally isolated (tables 3.5.1 to 3.5.5). Chi-square analysis of contingency tables indicated that all isolates were primarily wound pathogens ($p<0.001$) although some infection did occur through non-wounded surfaces. Virulence (the diameter of the lesion) of isolate LB to cultivar 'Lady Betty Balfour' at days 4 and 7 was analysed (table 3.5.6), showing that at day 7 the effect of scrape wounding is clearly different ($p=0.05$) from the other two leaf disc treatments. The effects of leaf disc treatment on lesion size after 7 days of the other four isolates are compared in tables 3.5.7 to 3.5.10. Scrape wounding (or either type of wounding for isolate MT) produces larger lesions ($p=0.05$) after 7 days than non-wounding.

Isolate aggressiveness towards cultivars after 7 days is shown in table 3.5.11. Lesion number was affected by both cultivar and isolate but there was no interaction indicating that each isolate was equally aggressive towards each particular cultivar. Analysis of this table with isolate MT and cultivar 'Montana' excluded (unpublished) showed isolates LB, EM, HD and RC were equivalently aggressive towards the four remaining cultivars.

Analysis of isolate virulence towards each cultivar (table 3.5.12) by two-way ANOVA indicated further differences in cultivar susceptibility (table 3.5.13). Differences in virulence between isolates are indicated by table 3.5.14.

Spore suspensions of isolate LB were equally aggressive towards wounded and non-wounded sepal discs (table 3.5.15), but results are of little use because of rapid physical deterioration and contamination of control discs.

3.6 GLASSHOUSE INFECTION

3.6.1 Koch's Postulates

Isolate LB caused all inoculated plants to wilt, although the time taken to wilt was extremely variable (table 3.6.1). Two other symptoms were noted; the petiole attached to the inoculated node and stem above and below the inoculated node blackened, and the leaf attached to the inoculation point usually wilted ("leaf wilt") before the plant wilted.

Phoma isolates grew from the rotted nodal tissue and lesion sites on the stem and petiole onto GSP. Isolates were identical to isolate LB after growth on PDA (spore size, colony colour and size).

3.6.2 Infection of Plant Organs

Lesions usually only developed on wounded plant surfaces except stems (table 3.6.2). Lesions did not develop on control plants (wounded/ nonwounded surfaces inoculated with T5 solution).

Table 3.6.1 Symptom development and disease progression[1] in cultivar 'Lady Betty Balfour'

Day	Plant 1	2	3	4	5
0		----------Inoculation[2]----------			
10	LW				
11	W				
25		W	LW		
30				LW	LW
41			W	W	W

(1) LW "leaf wilt" W "wilt"

(2) infection via wounded leaf axils
inoculum spore/ mycelium mixture

(3) control plants showed no symptoms

n=5

Table 3.6.2 Effect of wounding plant organs on lesion production: 'Lady Betty Balfour' plants inoculated with isolate LB spore suspension

Treatment	Plant Part	Day 7	14	21
percent infection sites with visible lesion beyond inoculation point				
Wounded	Leaf	80	80	87
	Petiole	33	87	87
	Axil	0	67	100
	Stem	0	0	0
Non-wounded	Leaf	0	0	7
	Petiole	0	0	0
	Axil	0	0	0
	Stem	0	0	0

(1) control plant parts inoculated wilth T5 solution did not develop symptoms

n=30

Table 3.6.3 Effect of covering infection sites on lesion production: 'Lady Betty Balfour' inoculated with isolate LB spore suspension

Treatment[1]	Day 7	10	14	21
percent infection sites with visible lesion beyond inoculation point				
Covered				
Wounded	72	72	80	92
Non-wounded	4	4	20	20
Uncovered				
Wounded	64	76	96	96
Non-wounded	4	8	12	12

(1) leaves covered with plastic bags for 48h after inoculation

(2) controls inoculated with T5 solution did not develop lesions

n=30

Table 3.6.4 Effect of wounding and covering on lesion size: 'Lady Betty Balfour' inoculated with isolate LB spore suspension

	Covered	Uncovered
	average diameter of lesions (mm)	
Non Wounded	5.4	6.3
Wounded	8.6	8.2

CV = 27.6

n=30

Table 3.6.5 Number of lesions on each cultivar after inoculation with corresponding fungal isolate

Isolate	Treatment	Day 7	10	14	21
		percent infection sites with visible lesion beyond inoculation point			
LB	Wounded	32	100	100	100
	Non-wounded	0	0	0	0
MT	Wounded	0	24	36	*
	Non-wounded	0	0	0	0
EM	Wounded	8	24	72	92
	Non-wounded	0	0	0	0
HD	Wounded	80	100	100	100
	Non-wounded	0	0	0	0
RC	Wounded	4	52	88	100
	Non-wounded	0	0	0	0

(1) * abscision of infection sites

n=25

Table 3.6.6 Size of lesion caused by fungal isolate on corresponding cultivar after 14 days

	Isolate LB	MT	EM	HD	RC
	average diameter of lesions (mm)				
	9.5	6.5	5.0	8.2	6.3
DNMRT p=0.05	a	b c	c	a b	c

n=25

Figure 3.6.1 Yellowing of infected 'Huldine' leaves

Leaf with developing lesion beginning to yellow (day21). Note yellowing only occurs where lesion has developed, not as a wound response

Figure 3.6.2 Progressive yellowing of leaves

By day 25 yellowing of infected leaves is quite pronounced

Figure 3.6.3 Complete yellowing of infected 'Huldine' leaves

Lowest pair of leaves wounded and inoculated, next pair wound control leaves (day 35)

Table 3.6.7 Abscission of infected leaflets from 'Montana' plants

	Plant number				
Day	1	2	3	4	5
7		All leaflets attached			
14					1
21	1	1	2	3	1
28	3	1	3	3	1

leaflets did not abscise in response to wounding - only leaflets with lesions abscised (leaf normally comprised of three leaflets)

3.6.3 Infection in glasshouse environment

Aggressiveness and virulence of isolate LB to cultivar 'Lady Betty Balfour' was not influenced by increased humidity during the first 48h of incubation. Chi-square analysis of the aggressiveness contingency table indicated that wounding was the only factor affecting infection ($p<0.001$). Two-way ANOVA analysis of virulence results also showed that wounding was the only factor affecting lesion size ($p<0.01$).

3.6.4 Glasshouse Infection

Aggressiveness and virulence of each isolate towards the cultivar from which it was originally isolated is recorded in tables 3.6.5 and 3.6.6. All isolates were wound pathogens. Some infection (4%) occurred at wound control sites inoculated with T5 solution. This was probably due to spores dripping off inoculated leaves above the control leaves during watering.

During this investigation it was noticed that infected leaves of 'Huldine' yellowed (figures 3.6.1, 3.6.2 and 3.6.3) and leafspots ceased to expand from approximately day 21. Infected leaflets and leaves of 'Montana' abscissed from either the petiole or stem from day 14 (table 3.6.7), when the leaf spot edge was still 8-10mm from the abscission zone. Yellowing or abscission did not occur to wounded control leaves. These effects were not observed with the other cultivars; after 21 days leafspots had usually expanded into the petiole of the infected leaf or leaflet.

3.7 LABORATORY FUNGICIDE TRIALS

3.7.1 Protective Fungicides

Minimum inhibitory concentration (MIC) values (lowest fungicide concentration which completely inhibited spore germination) of the protective fungicides are recorded in table 3.7.1. ANOVA analysis of spore germination of 'Bravo', 'Euparen' and 'Difolatan' spore suspensions at 0.1 ug/ml suggested that 'Bravo' was the more effective fungicide (table 3.7.2), though considerable variation occurred between replicates. Estimation of the ED_{50} concentrations by MLP (maximum likelihood program) or LR (linear regression) supported this conclusion although the standard errors were too large to statistically separate the ED_{50} values.

'Bravo' completely inhibited spore germination of all isolates at an AI concentration of 1 ug/ml. Isolate association based on spore germination at 0.1 ug/ml is shown in table 3.7.3 although results must be interpreted with caution as there was considerable variation between replicates.

3.7.2 Systemic Fungicides

MIC values (minimum fungicide concentration which completely inhibited growth) for systemic fungicides are shown in table 3.7.4. Of the six fungistatic

Table 3.7.1 Activity of protective fungicides

Fungicide	MIC[1] for spore germination inhibition (μg/ml)
Bravo	1
Euparen	1
Ronilan	>1000
Manzeb	100
Maneb	10
Thiram	10
Zineb	1000
Copper Oxychloride	1000
Sulphur	>1000
Rovral	>1000
Sportak	>1000
Difolatan	1
Captan	10
Saprol	100

(1) Minimum inhibitory concentration n=5

Table 3.7.2 Inhibition of spore germination by 0.1 μg/ml protectant fungicide

	Fungicide		
	Bravo	Euparen	Difolatan
Percent germination	10.4	67.2	84.8
DNMRT p=0.05		a	a
ED_{50}	0.043(MLP)	0.069(LR)	0.09(LR)
SE of ED_{50}	1.17	1.80	2.06

CV = 28.7% n=5

Table 3.7.3 Effect of 'Bravo' on spore germination

Bravo Concentration	Isolate				
	LB	MT	EM	HD	RC
	percent spore germination				
1 μg/ml	0	0	0	0	0
0.1 μg/ml	64.8	3.8	92.2	34.6	63.4
DNMRT p=0.05	a			a	a
	b		b		b

CV = 41.7% n=5

Table 3.7.4 Activity of systemic fungicides

Fungicide	MIC to inhibit mycelial growth (*u*g/ml)
Kausmin	>100
Benlate	10
Bavisitin	10
Delsene	10
Topsin-M	>100
Ronilan	>100
Sportak	10
Corbel	1
Calixin	>100
Saprol	>100
Baycor	>100
Topas	>100
Tilt	10
Bayleton	>100
BAS 9018	>100
Calirus	>100

n=3

Table 3.7.5 Effect of 'Corbel' on isolate colony diameter

Isolate	Corbel concentration (*u*g/ml AI) 10	1	0.1	0.01
	percent reduction in colony diameter after 7 days			
LB	100	91	75	23
MT	100	83	32	5
EM	100	87	62	28
HD	100	89	63	3
RC	100	90	70	14

n=3

Table 3.7.6 Isolate association based on overall response to 'Corbel'

Isolate	LB	EM	RC	HD	MT
DNMRT p=0.05					

Table 3.7.7 Effect of 'Bravo'/ 'Corbel' mixtures on spore germination of isolate LB

	Corbel concentration (*u*g/ml)			
Bravo concentration (*u*g/ml)	10	1	0.1	0
	percent spore germination			
10	0	0	0	0
1	0	0	0	0
0.1	88.3	93.3	94.6	91.8
0	95.2	96.8	97.6	96.2

CV = 4.5%

n=5

Table 3.7.8 Effect of 'Bravo'/ 'Corbel' mixtures on colony diameter of isolate LB

	Bravo (*u*g/ml)				
Corbel (*u*g/ml)	10	1	0.1	0.01	0
	colony diameter after 7 days (mm)				
10	5	5	5	5	5
1	6.7 N	6.3 N	7.3 N	7.3 N	6.3
0.1	33.7 A	34.7 A	31.3 A	25.0 N	23.7
0.01	48.0	50.7	56.3N	56.3	57.7
0	47.3	51.7	54.7	57.3	57.3

CV = 2.4% n=3

(1) 5mm = diameter of original inoculum

(2) N no interaction: A antagonistic

(based on DNMRT p=0.05 associations)

Table 3.8.1 Effect of fungicide and spray/ inoculation sequence on lesion production

Sequence	Spray	Day 7	14	21
		percent lesions at inoculation sites		
A: Inoculate/	Water	98	98	98
Spray	Fungicide	5	10	10
B: Spray/	Water	65	67	86
Inoculate	Fungicide	0	5	8

(1) chi-square test

day	ns
treatment order	$p<0.01$
fungicide	$p<0.001$

n = 60

Table 3.8.2 Effect of fungicide and spray/ inoculation sequence on lesion size

	Sequence			
	A: Inoculate/ Spray		B: Spray/ Inoculate	
Spray	Water	Fungicide	Water	Fungicide
Lesion diameter (mm)	8.6	5.4	6.6	4.3

CV = 25.1%

n = 60

formulations 'Sportak', 'Tilt' and 'Corbel' demonstrated fungicidal activity (100, 100 and 10 ug/ml respectively). Table 3.7.5 shows the percentage reduction in colony diameter with respect to 'Corbel' concentration. Isolate association based on overall response to 'Corbel' concentrations is shown in table 3.7.6.

3.7.3 Fungicide Interactions

Two-way ANOVA analysis of the effect of 'Bravo'/ 'Corbel' combinations on spore germination indicated no effect ($p=0.15$) due to 'Corbel' or any interaction ($p=0.75$) (table 3.7.7). The only effect was due to 'Bravo' ($p<0.01$).

Significant decreases in colony diameter due to 'Corbel', 'Bravo' and combination (all $p<0.001$) were observed for some fungicide concentrations (table 3.7.8). 'Corbel' did not inhibit mycelial growth at concentrations less than 0.01 ug/ml; the observed inhibition was due to 'Bravo' (10 and 1 ug/ml). 'Bravo' has no effect on the activity of 'Corbel' at 'Corbel' concentrations less than 1 ug/ml. Antagonism between the two fungicides was apparent at some concentration combinations.

3.8 GLASSHOUSE FUNGICIDE TRIAL

Aggressiveness and virulence of isolate HD to cultivar 'Huldine' after appropriate fungicide treatment are shown in tables 3.8.1 and 3.8.2. Chi-square analysis of the aggressiveness contingency table indicated the fungicide mixture significantly ($p<0.001$) decreased isolate agressiveness. The sequence of inoculation/ spraying was also significant ($p<0.01$) suggesting the formulation was a better 'protectant' than 'eradicant'. These conclusions were supported by two-way ANOVA analysis of lesion diameter at day 14 (virulence). Significantly ($p<0.01$) smaller lesions developed on plants sprayed with fungicide; smaller lesions developed on plants sprayed 7 days before inoculation ($p<0.05$).

3.9 TOXIN PRODUCTION

3.9.1 *In Vitro* Toxin Production by Isolate LB

Concentration of toxin in the culture broth increased from day 5 to day 11. There was no increase after day 11. Mycelial dry weight decreased from day 5 to day 9 before increasing again (table 3.9.1).

3.9.2 *In Vitro* Toxin Production by Isolates

Toxin production and mycelial dry weight of five New Zealand isolates after 11 days growth are recorded in table 3.9.2

Table 3.9.1 Toxin and mycelium production *in vitro* by isolate LB

	Day				
	5	7	9	11	14
Toxin Yield[1]	4.23	14.1	15.2	19.0	19.3
DNMRT p=0.05					
Mycelium[2]	1.66	1.35	1.25	1.44	1.43
DNMRT p=0.05		a		a	a

(1) mg crude toxin / 70 ml acidified culture filtrate

(2) dry mycelium weight (g)/ 250ml culture filtrate

Table 3.9.2 Toxin and mycelium production *in vitro* by *P. clematidina* isolates after 11 days

	Isolate				
	LB	EM	HD	RC	MT
Toxin Yield[1]	19.0	19.1	18.2	16.3	2.2
DNMRT p=0.05					
Mycelium[2]	1.63	1.09	1.72	1.72	1.08
DNMRT p=0.05	a	b	a	a	b

(1) mg crude toxin / 70ml acidified culture filtrate

(2) dry mycelium weight (g) / 250ml culture filtrate

Table 3.9.3 Identification of toxin isolated from leaf lesions by TLC analysis

Band No.	Visible colour[1]	UV colour	R_f
acetone-ethyl acetate-water: silica gel 1B			
1	*	Blue	0.63
2	Yellow	Green-Yellow	0.54
cyclo hexane-chloroform- acetic acid: silica gel 1B			
1	Yellow	Green-Yellow	0.25
2	*	Blue	0.08

(1) * Not visualised

Table 3.9.4 Weight of crude toxin in infected leaf discs

Extract No.	Weight of leaf disc tissue (g)	Weight of toxin (mg)	mg/ g[1]
1	1.17	0.60	0.51
2	1.13	0.80	0.70
3	0.68	0.46	0.68

(1) mg crude toxin/ 1g leaf tissue (fresh)

Mean (mg/g)	0.63
Standard Error	0.06

Figure 3.10.1 Toxin ultraviolet spectrum

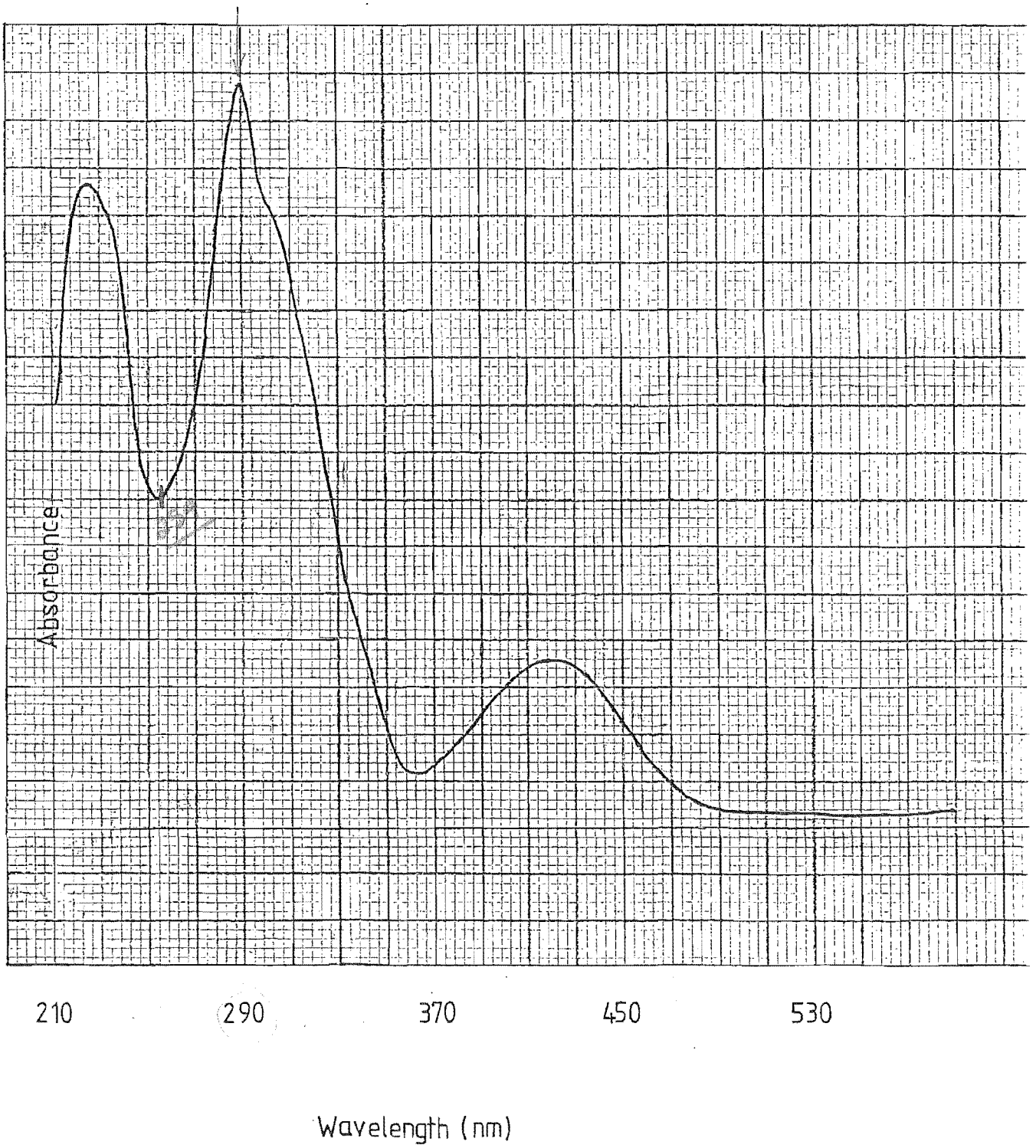


Table 3.10.1 Toxin solublity

Solvent	Toxin soluble in 100% solvent	Toxin soluble in 2% (v/v) solvent
ethanol	Yes	No
n-propanol	No	No
propan-2-ol	No	No
2-methoxyethanol	Yes	No
acetone	Yes	Yes

Table 3.10.2 Toxin purification: TLC analysis of primary chloroform extract

Band No.	Visible colour(1)	UV colour	R_f
acetone-ethyl acetate-water : silica gel 1B			
1	*	Blue	0.60
2	Yellow	Green-Yellow	0.52
3	*	Blue	0.46
4	*	Yellow	0.42
5	*	Blue	0.34
6	*	Blue	0.21

(1) * Not visualised

Table 3.10.3 Toxin purification: TLC analysis after Na_2CO_3 purification

Band No.	Visible colour[1]	UV colour	R_f
acetone-ethyl acetate-water: silica gel 1B			
1	*	Blue	0.62
2	Yellow	Green-Yellow	0.53
3	*	Blue	0.23
cyclo hexane-chloroform-acetic acid: silica gel 1B			
1	Yellow	Green-Yellow	0.25
2	*	Blue	0.11

(1) * Not visualised

Table 3.10.4 Toxin purification: TLC analysis after primary methanol purification

Band No.	Visible colour[1]	UV colour	R_f
acetone-ethyl acetate-water : silica gel 1B			
1	*	Blue (faint)	0.64
2	Yellow	Green-Yellow	0.52
cyclo hexane-chloroform-acetic acid: silica gel 1B			
1	Yellow	Green-Yellow	0.26
2	*	Blue (faint)	0.10

(1) * Not visualised

Table 3.10.5 Toxin purification: TLC analysis after secondary methanol purification

Band No.	Visible colour[1]	UV colour	R_f
acetone-ethyl acetate-water: silica gel 1B			
1	Yellow	Green-Yellow	0.52
cyclo hexane-chloroform-acetic acid: silica gel 1B			
1	Yellow	Green-Yellow	0.25

(1) * Not visualised

Table 3.10.6 Toxin purification: two dimensional chromatography

Spot No.	Visible colour	UV colour	R_f
acetone-ethyl acetate-water: silica gel B			
1	Yellow	Green-Yellow	0.59
cyclo hexane-chloroform-acetic acid: silica gel B			
1	Yellow	Green-Yellow	0.19

3.9.3 *In Vivo* Toxin Production by Isolate LB

Toxin isolated from leaf-spots was identified by R_f value after thin layer chromatography (table 3.9.3) and green fluorescence when illuminated with 365nm monochromatic UV light (see section 3.10.4). Toxin was weighed and weight per gram of fresh leaf tissue calculated (table 3.9.4).

3.10 PURIFICATION AND CHARACTERISATION OF TOXIN

3.10.1 Basic Chemical Nature

Toxin was isolated and partially purified from culture broths or leaf spots by Lepoivre's (1982a) method, suggesting a carboxylic acid nature. An ethanolic solution of toxin decolourised an acetone solution of potassium permanganate, gave a negative reaction with Fehling's solution and a red ferric reaction with an ethanol solution of ferric chloride. Toxin solution decolourised on addition of NaOH. These characteristics are identical to those reported for ascochitine by Oku and Nakanishi (1963).

3.10.2 Solubility

Toxin (2.10.2.2) completely dissolved in 100% ethanol or acetone to produce 500 *u*g/ml solutions. After dilution of these solutions to 2% (v/v) solvent concentration, only aqueous acetone retained toxin in solution (table 3.10.1). The initial range of toxin concentrations assayed was limited by this low toxin solubility.

3.10.3 Ultraviolet Spectrum

Two UV absorption maxima (224 and 289 nm) and one in the the visible spectrum (420nm) were detected for a methanol toxin solution (figure 3.10.1). Oku and Nakanishi (1963) reported absorption maxima of 220, 286, and 415 nm for an 'alcoholic solution' of ascochitine.

3.10.4 Thin Layer Chromatography

Each successive purification step produced a purer toxin preparation, evidenced by fewer bands on TLC plates (tables 3.10.2 to 3.10.5). Toxin was identified by yellow colour in visible light, green-yellow fluorescence when illuminated with UV light, and R_f values of *c* 0.52 and *c* 0.25 when chromatographed in the two solvent systems (acetone-ethyl acetate-water or cyclo hexane-chloroform-acetic acid) on silica gel 1B. Very small levels of contaminant existed in the toxin preparation after primary methanol purification (faint blue band in table 3.10.4). The crystalline powder obtained after secondary methanol purification chromatographed as a single spot (optical, UV) in both solvent systems (3.10.5). Two dimensional chromatography of this powder on silica B still visualised (optical, uv) as one spot (table 3.10.6). Exposure to sublimed iodine vapour, or spraying with sulphuric acid and heating still only visualised one spot.

Figure 3.10.2 Toxin HPLC (methanol:acetic acid)

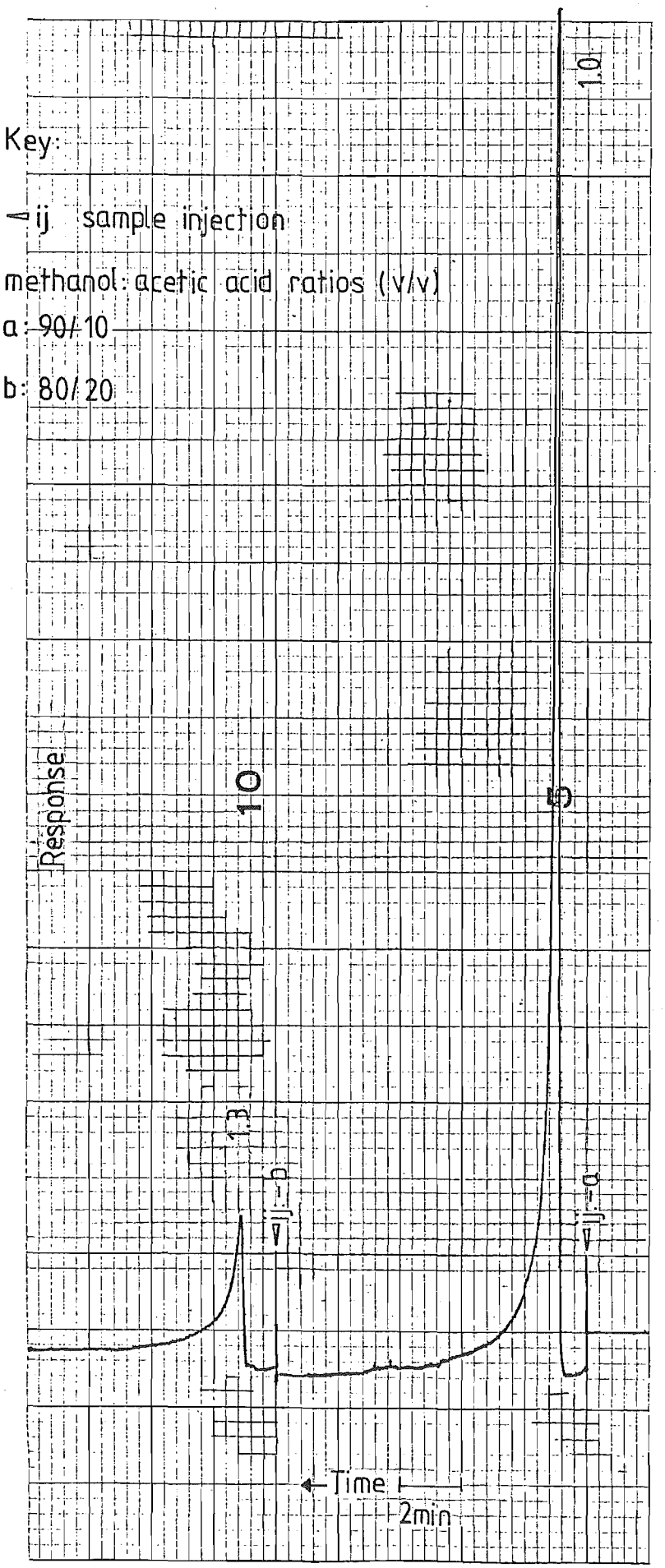


Figure 3.10.3 Toxin HPLC (methanol: trifluoroacetic acid)

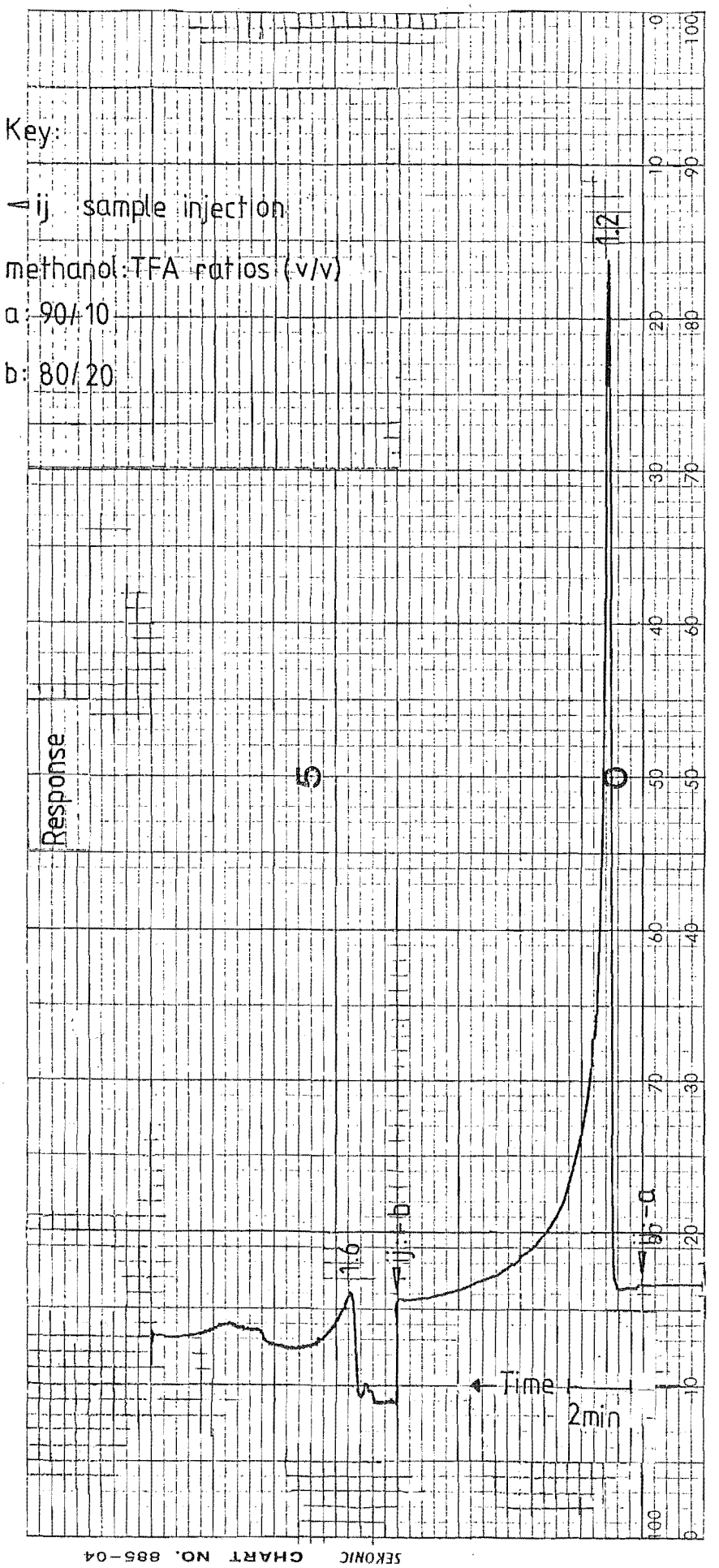


Figure 3.10.4 Toxin HPLC (acetonitrile: orthophosphoric acid)

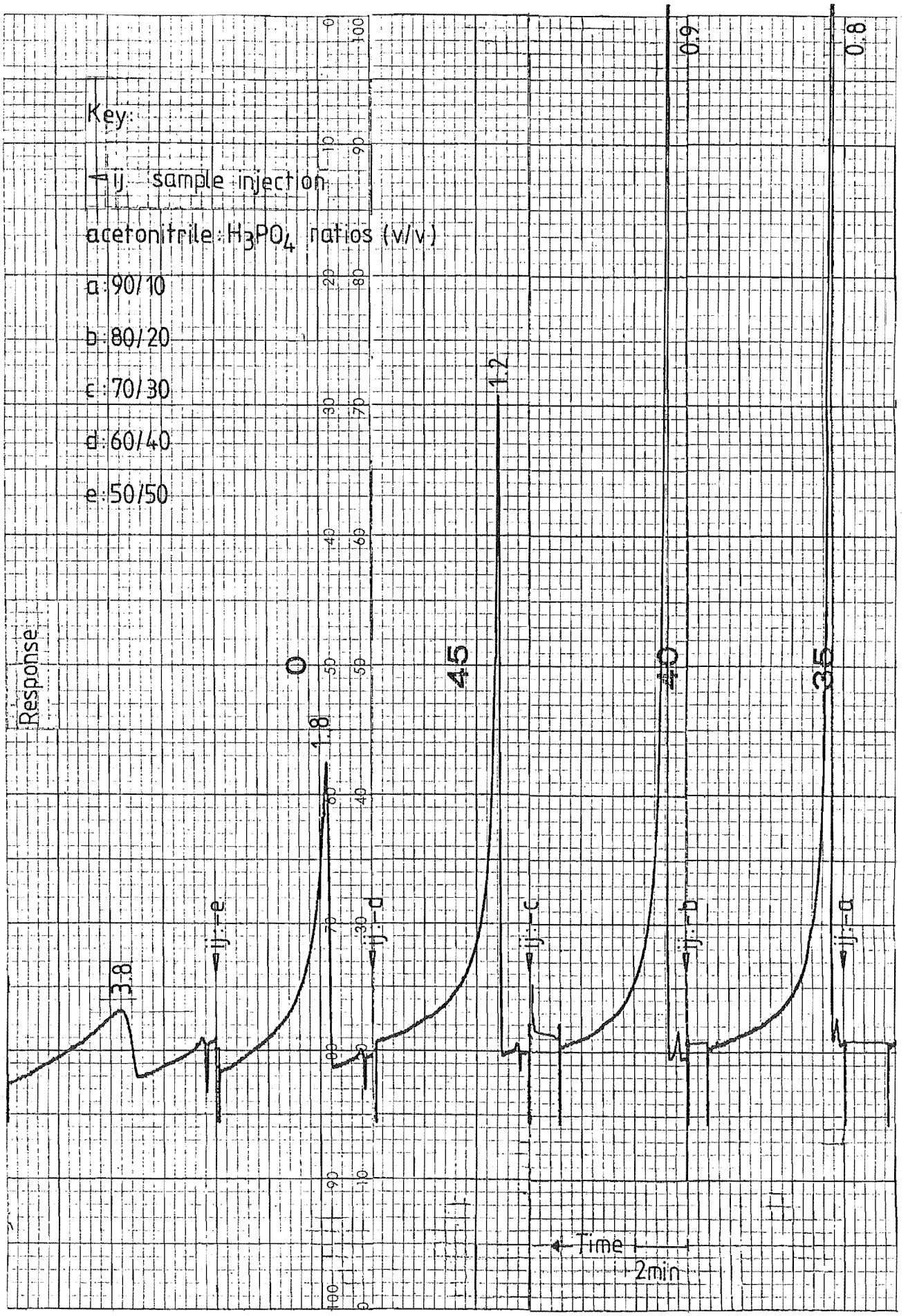
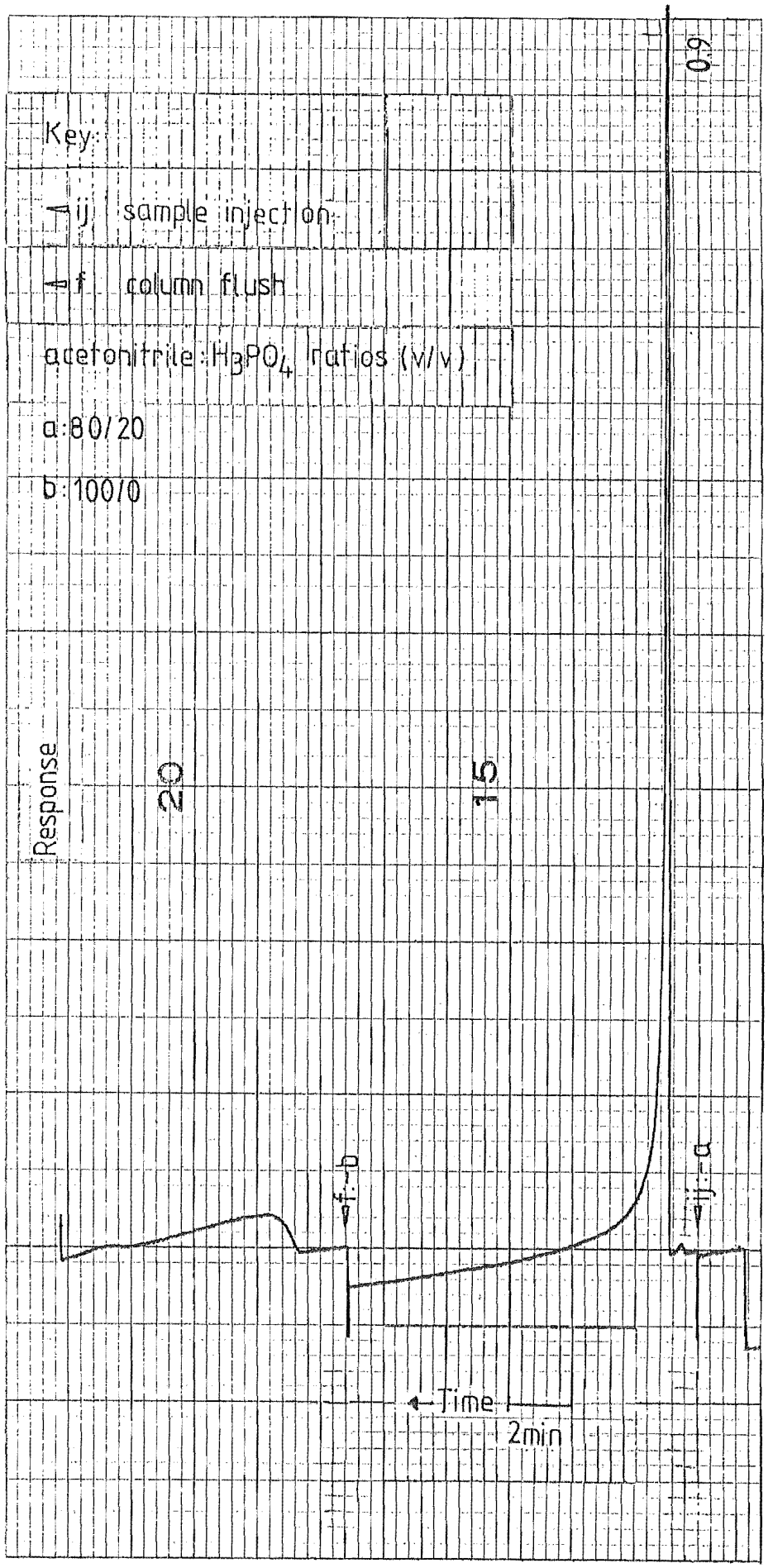


Figure 3.10.5 Toxin HPLC (acetonitrile: orthophosphoric acid)



3.10.5 High Performance Liquid Chromatography

3.10.5.1 Methanol: Acetic Acid. Resolution of toxin in 90:10 (v/v) methanol: 0.8% acetic acid was poor, travelling on the solvent front with considerable tailing. Decreasing the methanol concentration to 80% gave a slightly longer retention time but less resolution (figure 3.10.2).

3.10.5.2 Methanol: Trifluoroacetic Acid. Toxin peak resolution was not improved by chromatography in 90:10 or 80:20 (v/v) methanol: 0.05% trifluoracetic acid (figure 3.10.3). No peak resolved if the methanol concentration was decreased to 70% in either methanol system.

3.10.5.3 Acetonitrile: Phosphoric Acid. With a 10cm column installed, sharp toxin peaks with some tailing resolved in acetonitrile: $1x10^{-2}$M orthophosphoric acid mixtures. Decreasing the solvent concentration increased toxin retention time, but also lowered peak resolution until, at 50% acetonitrile no peak resolved (figure 3.10.4). Toxin chromatographed in 80:20 acetonitrile: acid resolved as a sharp 0.9min peak with some tailing. No other peaks resolved in the toxin peak tail, nor when the column was flushed with 100% acetonitrile (figure 3.10.5).

3.11 DEVELOPMENT OF TOXIN BIOASSAY

3.11.1 Choice of Assay

Results from the 'leaching solution' assay were not statistically different (ANOVA analysis) (table 3.11.1). The 'bathing solution' assay gave results with significant differences (table 3.11.2) and was developed further.

3.11.2 Assay Parameters

3.11.2.1 Solvent Effect. Toxin induced electrolyte leakage was independent of solvent concentration (table 3.11.3). This suggests that there is no interaction between toxin and solvent and the net response of leaf tissue to toxin is solvent response subtracted from overall response.

3.11.2.2 Time Response Curves. The 'solvent response' was subtracted from the total solution conductivity (figure 3.11.1) to give the 'toxin response'. The shape of the time response curve of each toxin concentration is shown by figure 3.11.2. The 20h response mean was selected to derive the dose response curve as this point was near the top of the linear part of the time response curves.

3.11.2.3 Toxin Concentration Response Curve. An approximately sigmoidal dose response curve was obtained from the 20h toxin response (figure 3.11.3). This curve was straightened with a $\log_{10}$ transformation and a regression line calculated (table 3.11.4) for sub-saturation toxin concentrations (figure 3.11.4).

3.11.2.4 Assay Saturation Concentration. The toxin concentration that saturated the dose response curve was 15 μg/ml (figure 3.11.4 and table 3.11.5).

Table 3.11.1 Conductivity of 'leaching solutions' after 24 hours

	Controls		Toxin concentrations (μg/ml)	
	water	solvent	10	25
Response				
mean (μS)	78.1	75.4	129.0	137.4
DNMRT				
p=0.05				
CV = 40.9%				n=5

Table 3.11.2 Conductivity of 'bathing solutions' after 24 hours

	Controls		Toxin concentrations (μg/ml)		
	water	solvent	1	10	25
Response					
mean (μS)	48.0	69.9	79.4	156.8	319.2
DNMRT					
p=0.05					
p=0.01					
CV = 13.1%				n=5	

Table 3.11.3 Effect of solvent concentration on induced electrolyte leakage

Solvent (%(v/v))	Mean response (μS) control	toxin	Mean net response (μS)	Standard error
5	75.8	141.6	65.8	10.79
10	104.8	212.0	107.2	23.54

F-test between the net mean responses was not significant $n = 5$

Table 3.11.4 Regression analysis of $\log_{10}$ net solution conductivity

Regression line calculated for net solution conductivity at toxin concentrations of 0, 5, 10 and 15 μg/ml

y = 0.0307x	standard errors	slope	0.0045	
		intercept	0.0416	
		t-test of slope	t = 6.8	p<0.001
		correlation coefficent	r = 0.85	p<0.001

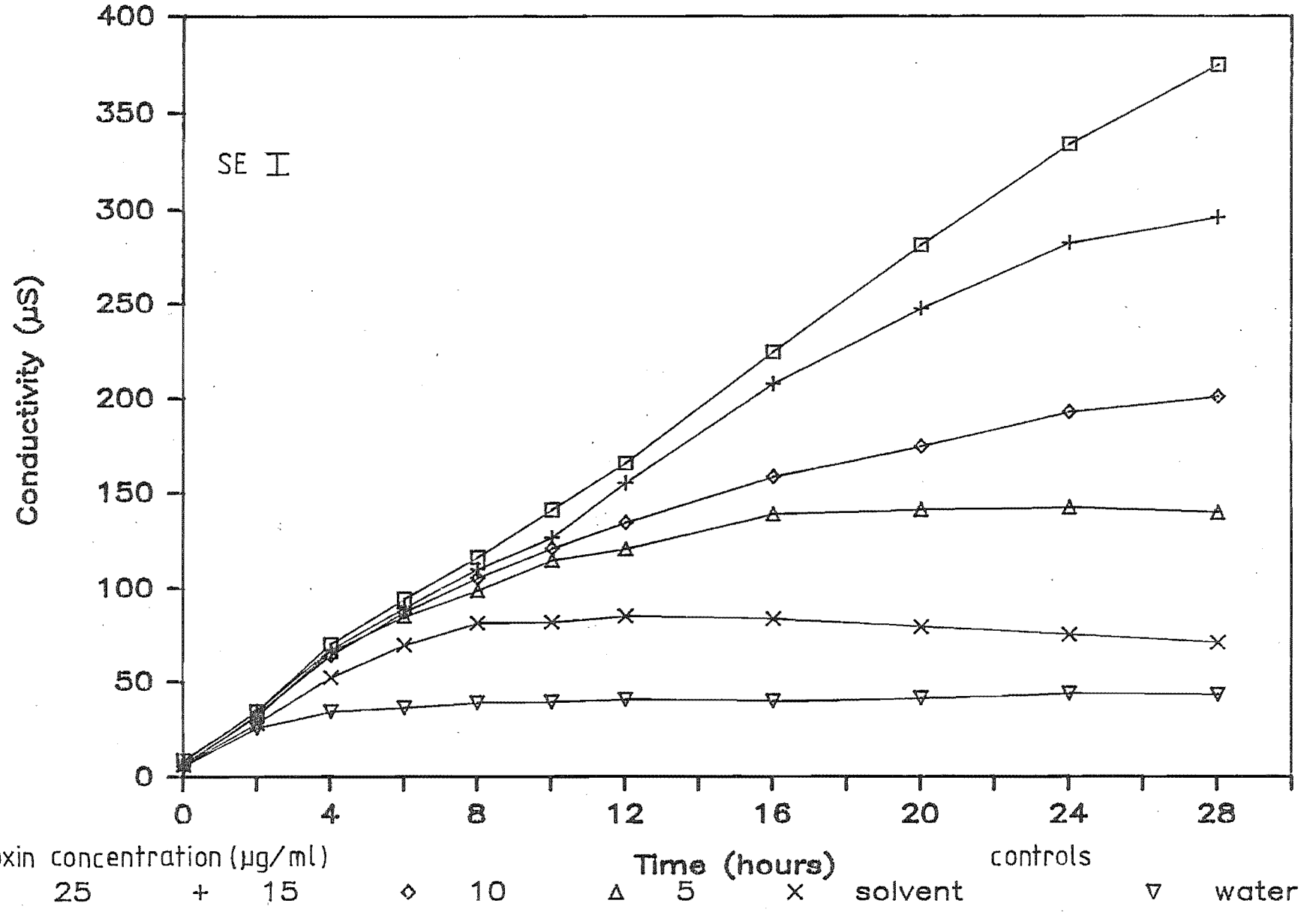


Figure 3.11.1 Conductivity of leaf disc bathing solutions

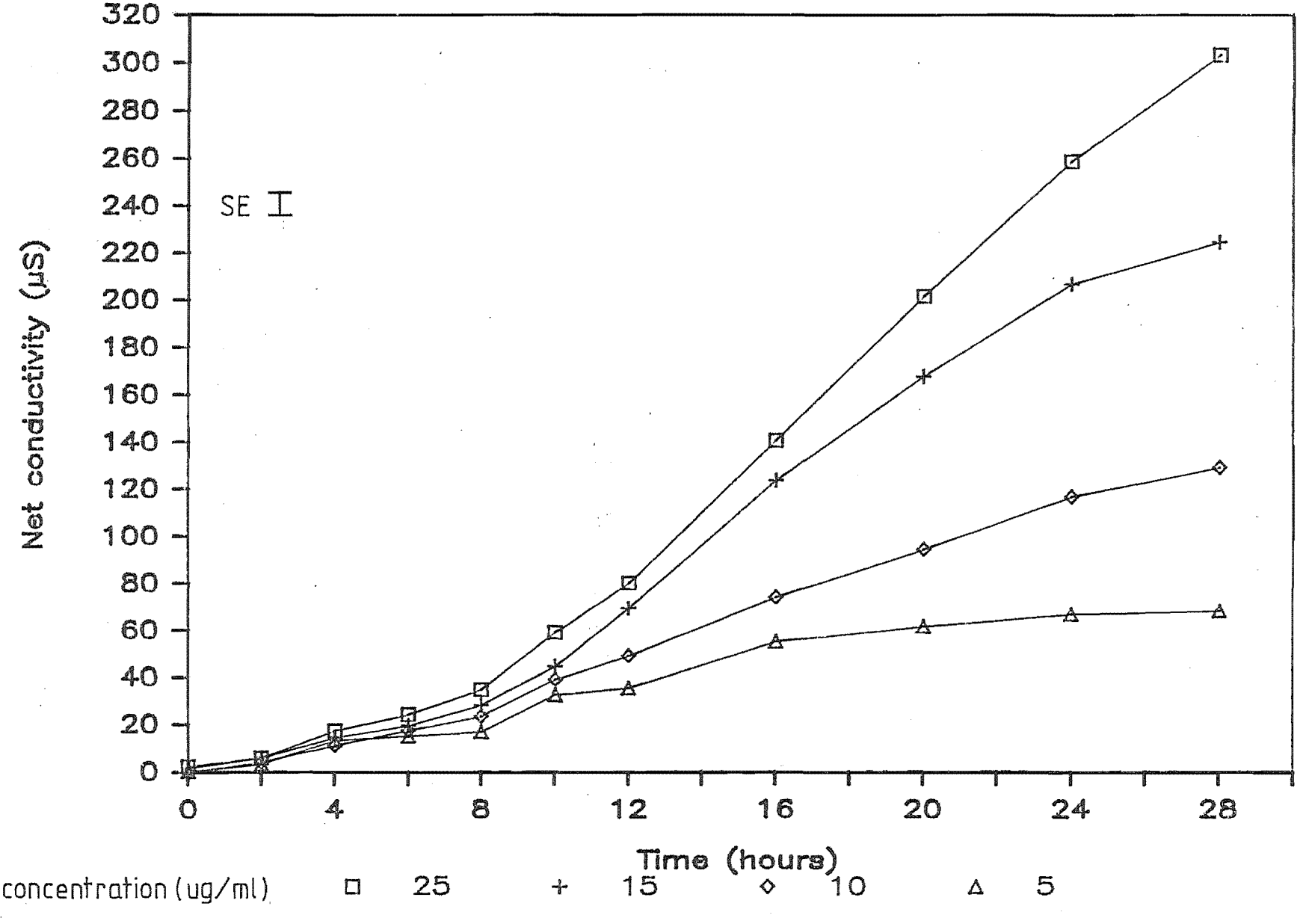


Figure 3.11.2 Net conductivity of bathing solutions

Figure 3.11.3 Net conductivity of bathing solutions after 20 hours

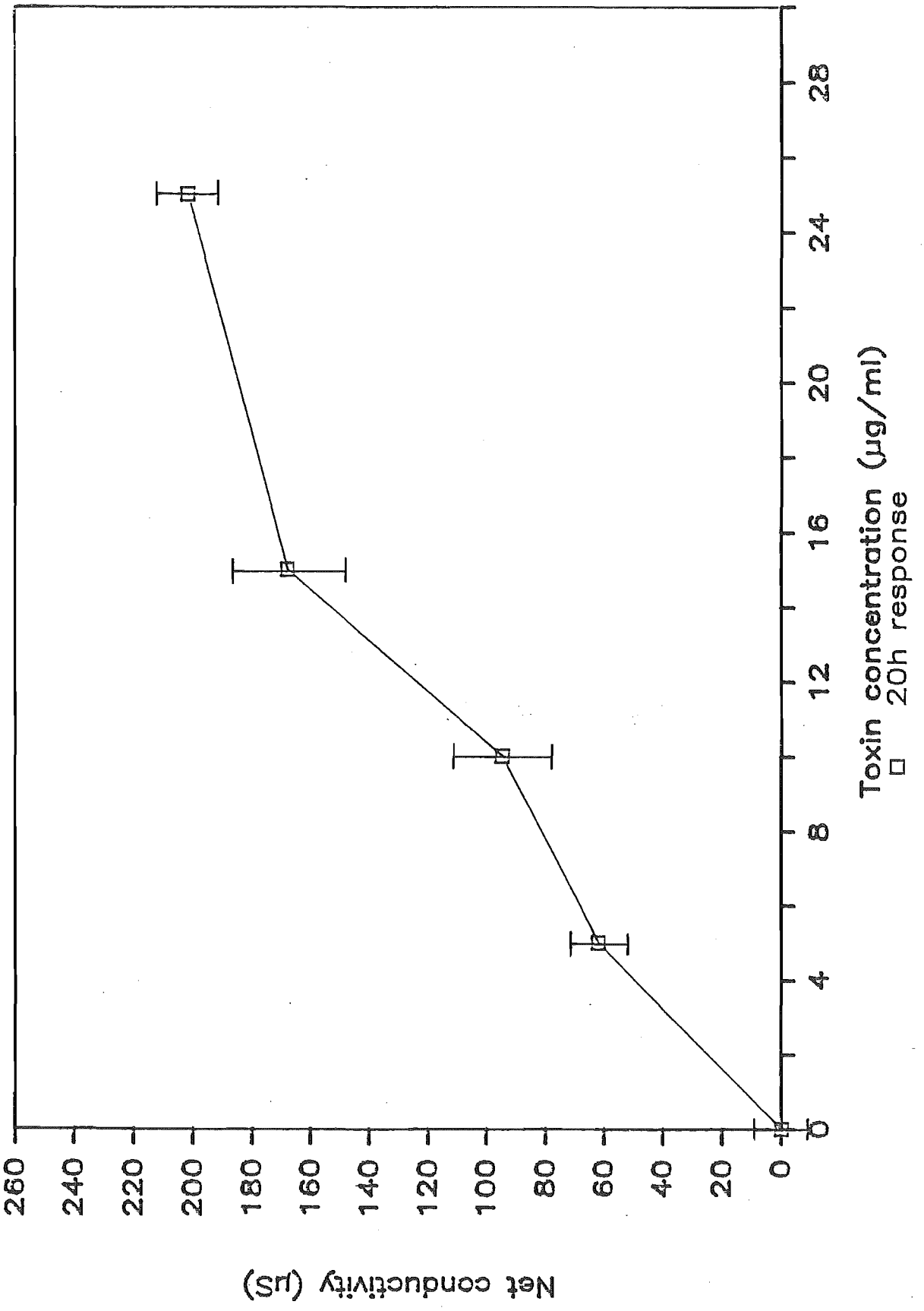


Figure 3.11.4 Saturation point of dose-response curve

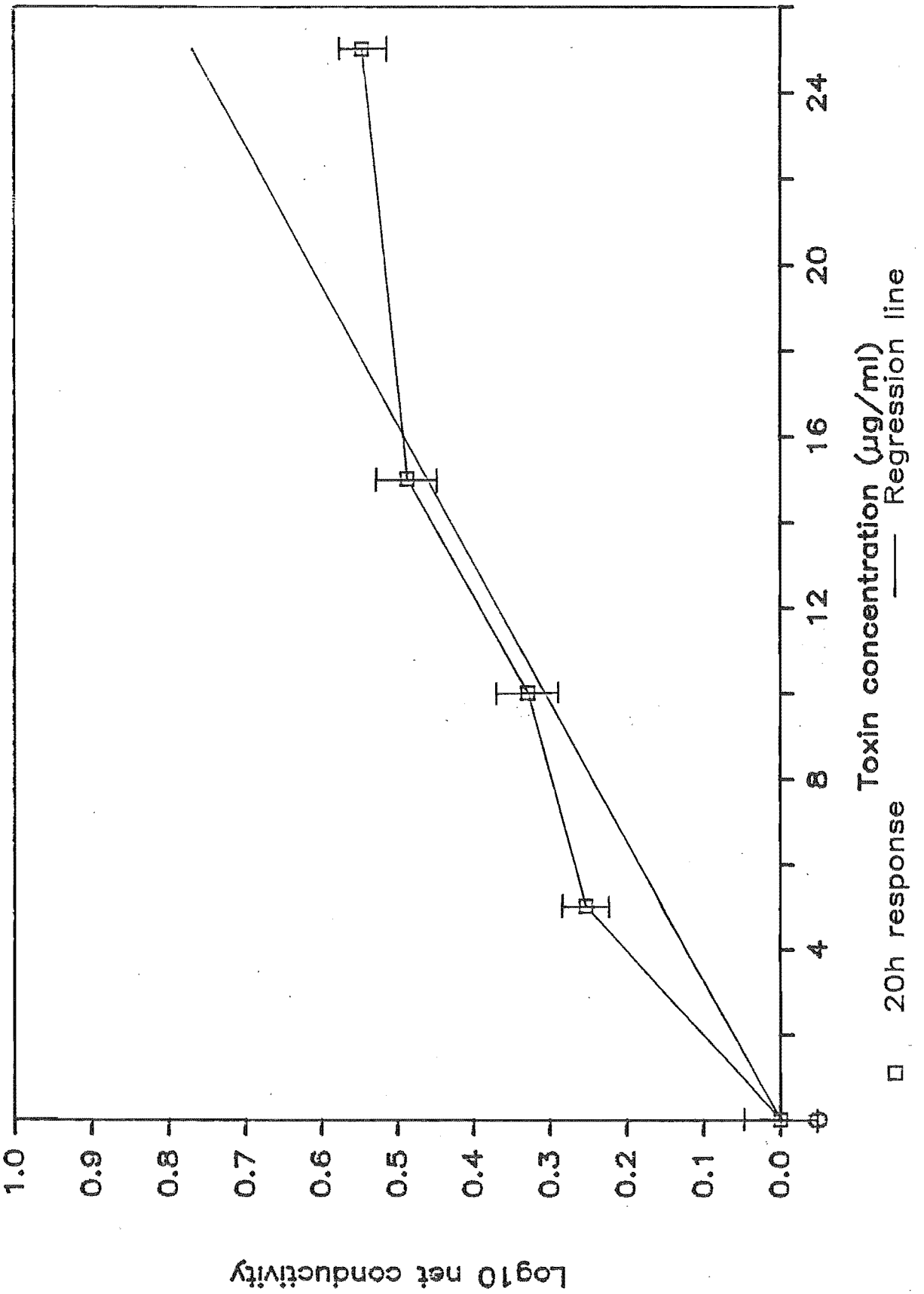


Table 3.11.5 'Bathing' solution conductivity after 20 hours

	Toxin concentration (μg/ml)				
	0	5	10	15	25
Response					
mean (μS)	81.6.	141.6	174.3	247.4	281.3
DNMRT					
p=0.05	_____				_____
CV = 22.9%					n=5

Table 3.11.6 Determination of minimum toxin concentration necessary to induce leakage

	Toxin concentration (μg/ml)				
	0	1	2.5	5	10
Response					
mean (μS)	72.9	78.3	91.6	127.0	216.4
DNMRT					
p=0.05	_____		_____		
p=0.01	_____		_____		
			_____		
CV = 16.9%					n=5

Regression line calculated for $\log_{10}$ transformed solution conductivities

y = 1.8469 + 0.04941x	standard errors	slope	0.0044	
		intercept	0.0224	
	t-test of slope		t = 11.22	p<0.001
	correlation coefficient		r = 0.92	p<0.001

Figure 3.11.5 Response curve to minimal toxin concentrations

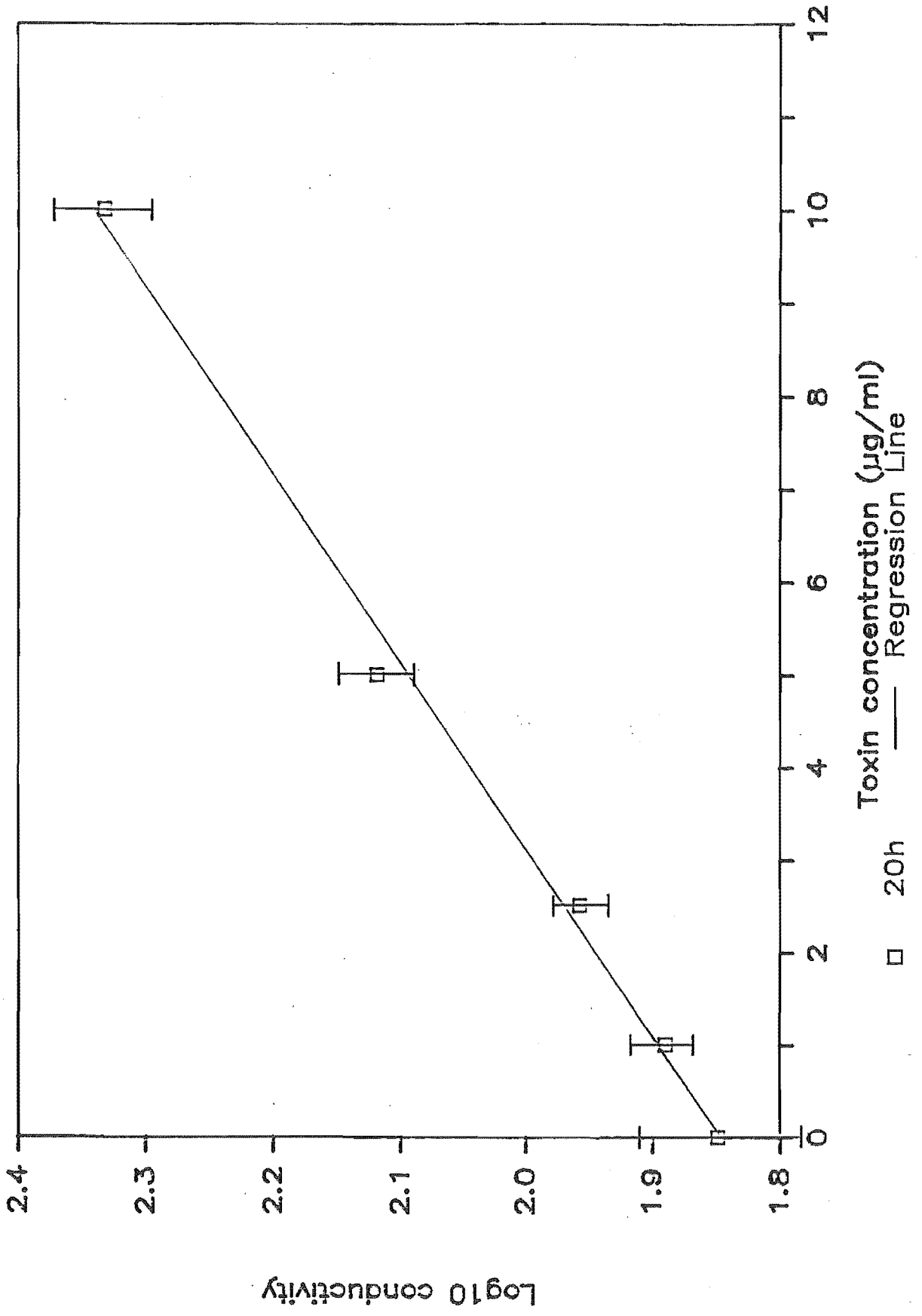


Table 3.11.7 Induced electrolyte leakage from five leaf discs

Cultivar	Response to 15 *u*g/ml toxin (*u*S)
'Lady Betty Balfour'	247.4
'Montana'	455.9

CV = 19.5% n=5

ANOVA indicated significant differences between means ($p<0.05$)

Table 3.11.8 Induced electrolyte leakage from three 'Montana' leaf discs

	Toxin concentration (*u*g/ml)					
	0	1	2.5	5	7.5	10
Response						
mean (*u*S)	67.7	68.9	68.2	61.9	197.0	230.3
DNMRT						
p=0.05	_____				_____	

CV = 22.7% n=5

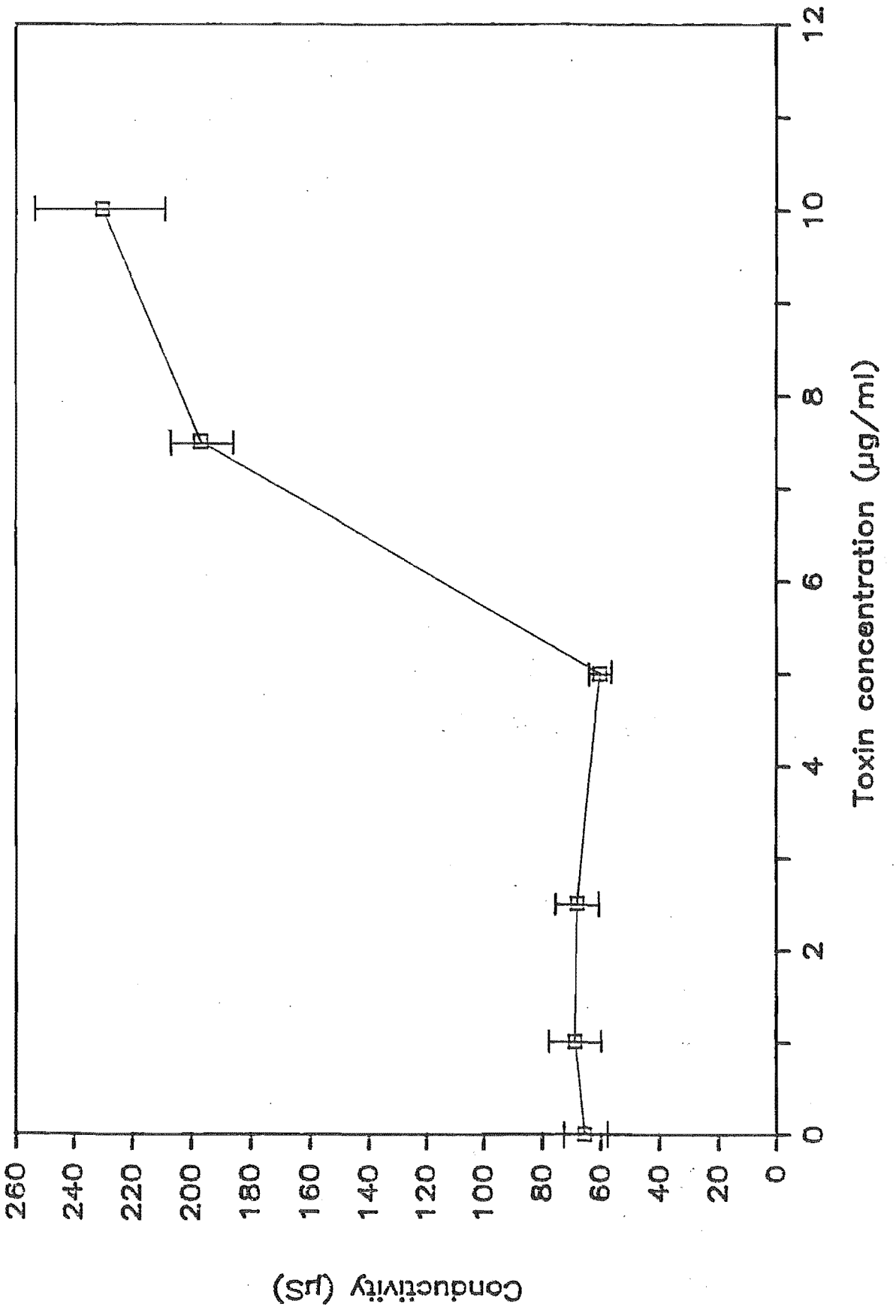
Table 3.11.9 Weight of cultivar leaf discs

	Cultivar						
	R	H	M	E	L	H	M
No. of discs	5	4	3	5	5	5	5
Mean weight (g)	.16	.16	.16	.17	.18	.20	.20
DNMRT							
p=0.05	_____						
p=0.01	_____						
				_____			

CV = 8.37% n=20

Cultivars 'Rouge Cardinal', 'Huldine', 'Montana',
'Ernest Markham', 'Lady Betty Balfour'

Figure 3.11.6 Dose-response curve of 'Montana' leaf discs



3.11.2.5 Minimum Toxin Concentration. Toxin concentrations of 1 and 2.5 ug/ml induced electrolyte leakage with responses on the dose response curve, but could not be statistically differentiated from the solvent response (figure 3.11.5. and table 3.11.6). The minimum toxin concentration necessary to induce endogenous electrolyte leakage was defined as 5 ug/ml as this response was statistically different from the solvent response.

3.11.2.6 Dosal Response of 'Montana' Leaf Tissue. Five 'Montana' discs leaked more than 5 'Lady Betty Balfour' discs (table 3.11.7). Three 'Montana' leaf discs assayed against toxin concentrations established the dose response curve of the resistant cultivar (figure 3.11.6). 'Montana' was resistant to induced electrolyte leakage by a toxin concentration of 5 ug/ml. At higher toxin concentrations leakage was induced (table 3.11.8).

3.11.2.7 Leaf Disc Weights. The number of leaf discs was adjusted so equivalent weights of leaf tissue were assayed (table 3.11.9).

3.12 TOXIN ASSAY

3.12.1 Ranking of Clematis Cultivars

The developed assay did not permit ranking of intermediate toxin sensitivity (figure 3.12.1). Only the mean responses of 'Lady Betty Balfour' and 'Montana' were significantly different from responses of the other three cultivars. 'Lady Betty Balfour' was most sensitive to toxin and 'Montana' most insensitive.

3.12.2 Effect of Cycloheximide on Electrolyte Leakage

Cycloheximide did not induce endogenous electrolyte leakage from 'Montana' leaf discs incubated with ascochitine. Cycloheximide did not affect controls, or affect the response of 'Lady Betty Balfour' leaf discs to ascochitine (table 3.12.1).

3.13 TOXIN STRUCTURAL EFFECTS

3.13.1 Visual Symptoms

Blackening of leaf discs was evident during the toxin assay (figures 3.13.1 and 3.13.2). The degree of blackening was related to toxin concentration. No blackening occurred in water or solvent control leaf discs. Black 'flecking' was noticed on 'Lady Betty Balfour' leaf tissue incubated with 1 and 2.5 ug/ml toxin solutions. 'Montana' leaf discs did not blacken until exposed to a toxin concentration of 7.5 ug/ml (figure 3.13.3).

Figure 3.12.1 Net conductivity of cultivar leaf disc bathing solutions

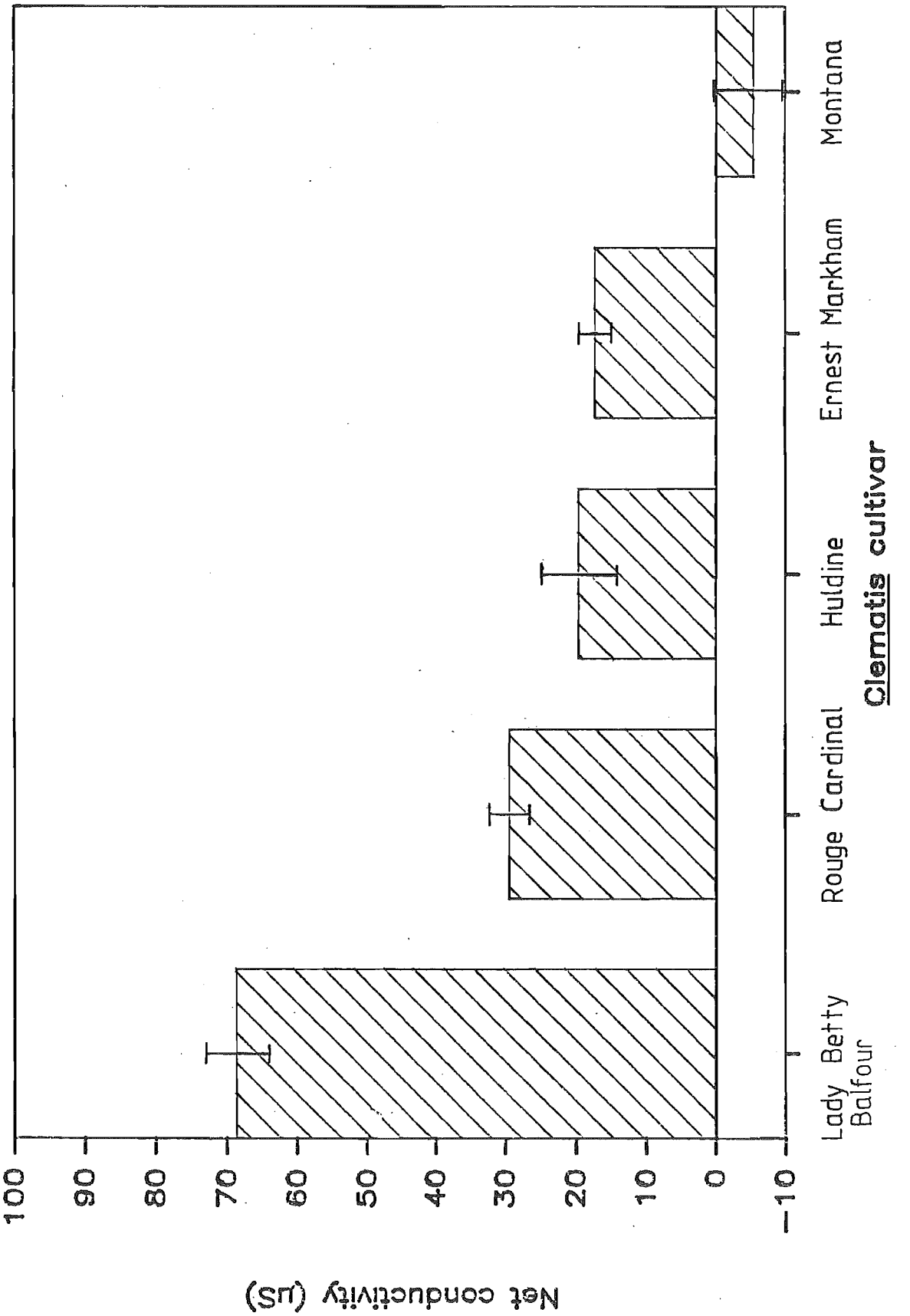


Table 3.12.1 Effect of cycloheximide on induced electrolyte leakage from leaf discs

Solutions	Cultivar 'Lady Betty Balfour'	'Montana'
	Mean conductivity of bathing solution after 20h (uS)	
Control	81.5 a	65.8 a
Cycloheximide	81.8 a	74.7 a
Toxin	126.7 b	73.2 a
Toxin + cycloheximide	123.9 b	75.8 a

CV = 12% n=5

Values follwed by the same letter in the same column are equivalent (DNMRT p=0.05)

Table 3.14.1 Antibiotic activity of toxin

Organism	Toxin concentration (*ug*/ml) 200	150	100	50	25	10	1	0
	inhibition zone surrounding disc (mm)[1]							
E. coli	7	0	0	0	0	0	0	0
B. subtilis	11	9	8	7	0	0	0	0

(1) inhibition diameter includes disc diameter of 6mm n=3

3.13.2 Effect at Cellular Level

Alterations to the cellular organisation of leaf cells was evident after exposure to toxin (figures 3.13.4 and 3.13.5), when compared with water (figures 3.13.6 and 3.13.7) and solvent controls (figures 3.13.8 and 3.13.9), and natural leaf tissue (figures 3.13.10 and 3.13.11). Cytoplasm appeared compressed and individual organelles (i.e. chloroplasts) could not be distinguished. This effect was observed along the entire length of the specimen, in all leaf cells (i.e. photosynthetic and conducting tissue).

Extensive damage to internal cellular membrane systems was evident in toxin damaged tissue (plates 13 and 14). Structural detail could not be resolved for organelle limiting membranes, nor for mitochondrial internal structures (cristae, stroma) (plates 13 c d and 14 a). Intact thylakoids are apparent inside the indistinct chloroplast envelope (plate 13) but some appear to be 'floating' probably due to loss of stromal matrix integrity (suggested by the granular appearance of the matrix). The plasmalemma (plate 13 b) and tonoplast (plate 13 a c) appear structurally intact and undamaged: this was also observed in dead leafspot cells (plate 15). The compressed cytoplasm (plate 13 c d) consisted of very coarse particles with a 'coagulated' appearance. Solvent control (plate 16) and fresh leaf (plate 17) tissue are included for comparison.

3.14 ANTIBIOTIC SPECTRUM OF TOXIN

Only *E. coli* and *B. subtilis* showed toxin sensitivity (table 3.14.1). The solvent had no visible effect on microbial growth.

3.15 TOXIN IDENTIFICATION

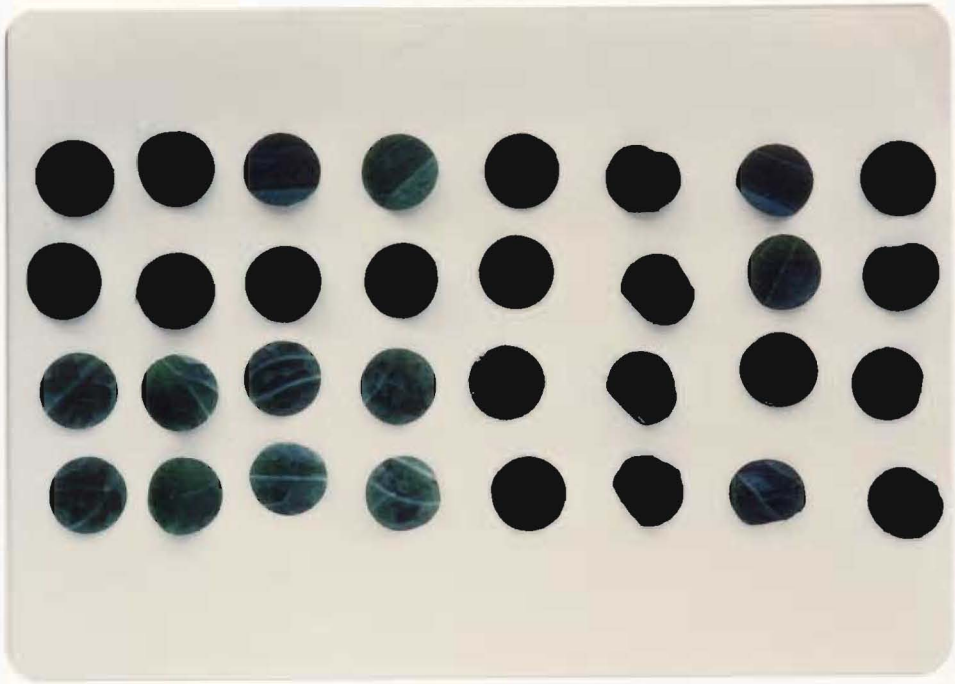
3.15.1 Nuclear Magnetic Resonance Spectrums

Analysis of the proton (^{1}H) spectrum (figures 3.15.3 and 3.15.4, table 3.15.1) by Dr M. H. G. Munro, Department of Chemistry, University of Canterbury, identified the toxin as ascochitine (figure 3.15.8). The carbon 13 (^{13}C) spectrum (figure 3.15.5) confirmed this identification, with heteronuclear correlation analysis (appendix 6) indicating the presence of two isomers.

3.15.2 Infrared Spectrum

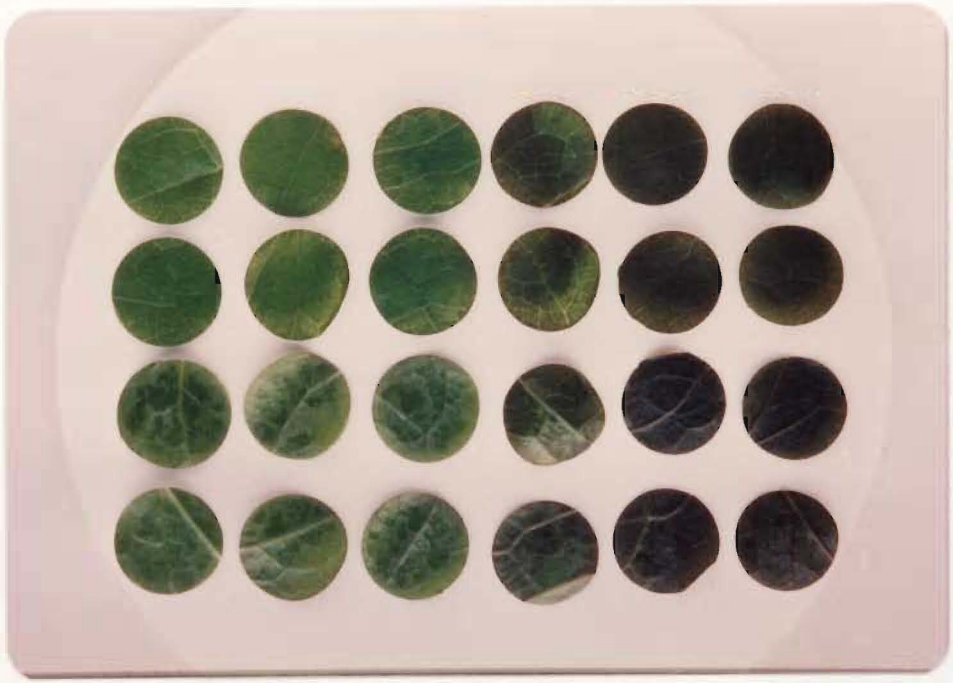
Toxin infrared transmittance in fused KBr (fig 3.15.1) and 'Nujol' (fig 3.15.2) were comparable with published ascochitine spectra (Oku and Nakanishi 1964b, Lepoivre 1982a).

Figure 3.13.1 Blackening of leaf discs during toxin assay



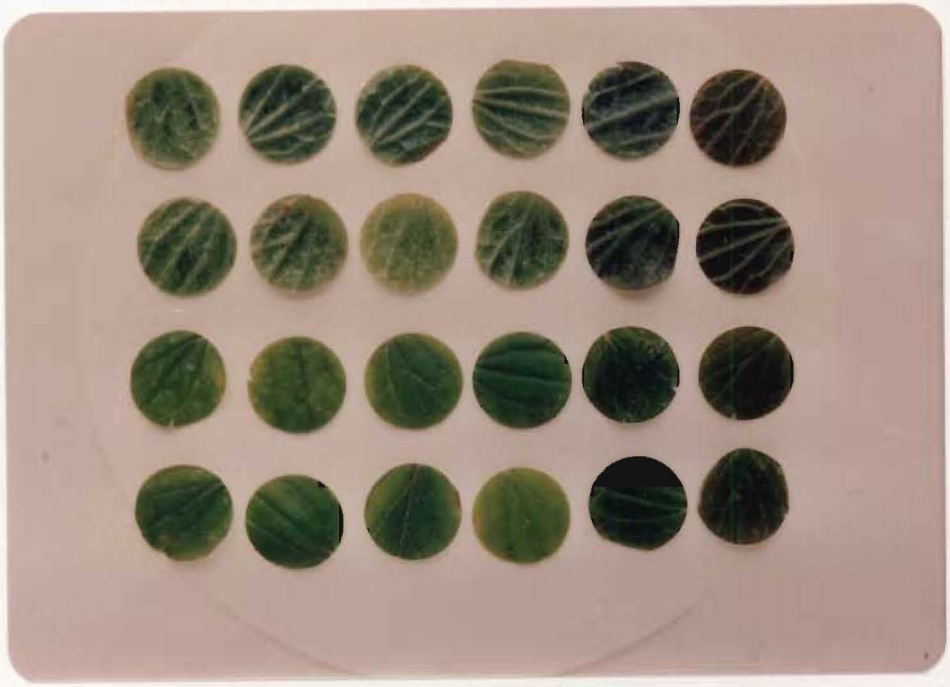
H_2O Ac 1 2.5 5 10 15 25
controls toxin concentration (μg/ml)

Figure 3.13.2 Effect of toxin concentration on leaf disc blackening



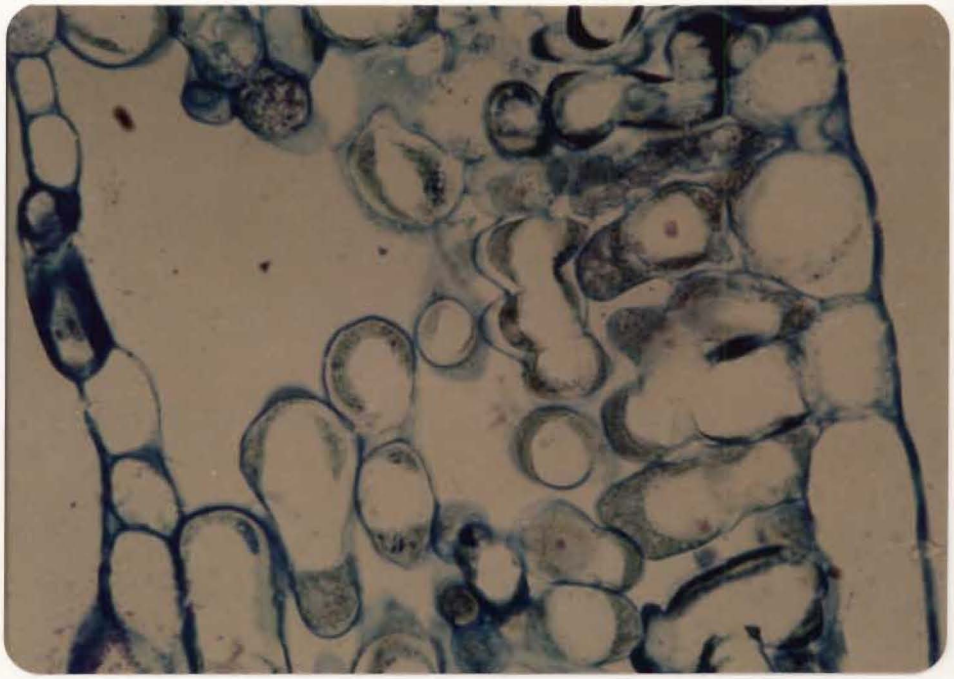
H_2O Ac 1 10 25 50 (t = 20h)
controls toxin concentration (μg/ml)

Figure 3.13.3 Appearance of 'Montana' leaf discs after toxin assay



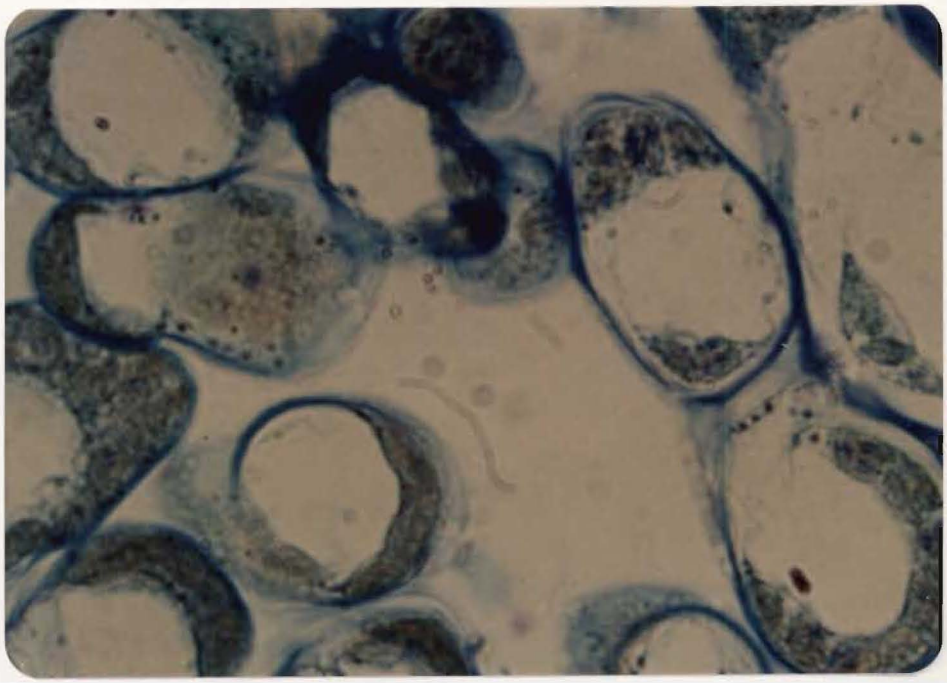
H_2O Ac 2.5 5 7.5 10
controls toxin concentation (μg/ml)
'Montana' leaf discs did not blacken after 20h exposure to 5 μg/ml toxin

Figure 3.13.4 Effect of toxin on cellular appearance



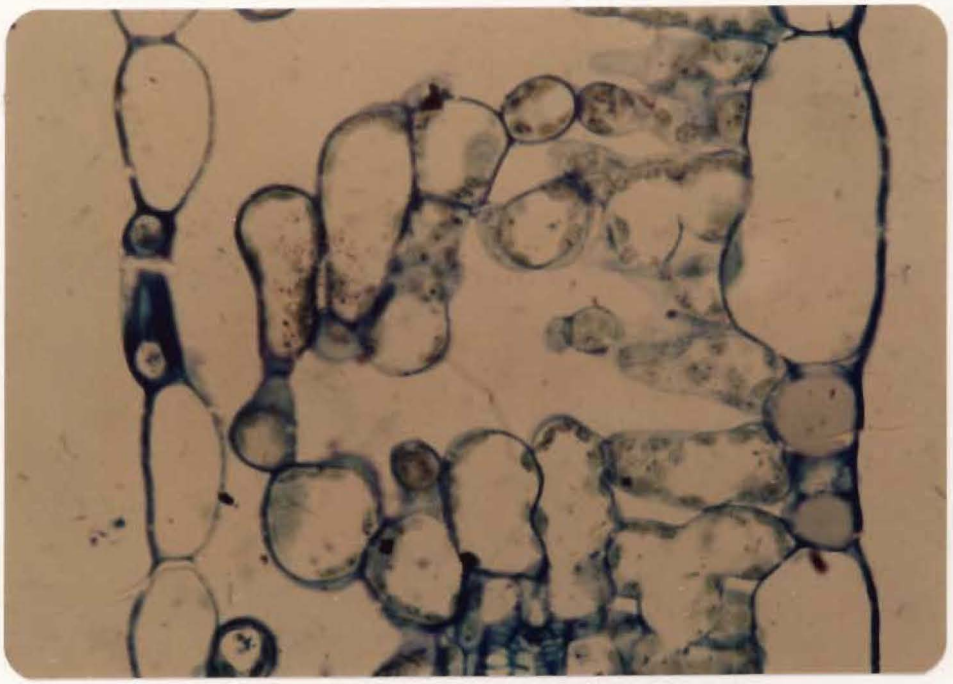
Cytoplasm of leaf cells appears compressed and homogenized with no organelles distinguishable (4 *u*m Spurrs/ Azur *II*) x400 (TS)

Figure 3.13.5 Detail of toxic effect on leaf cells



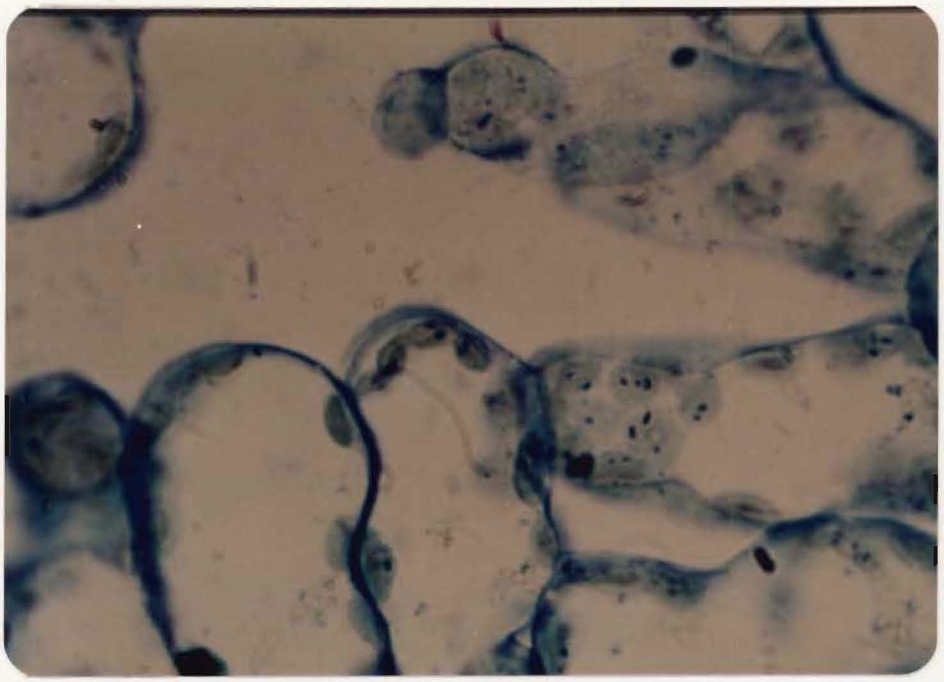
Chloroplasts can not be identified in the amorphous cytoplasm (4 *u*m Spurrs/ Azur *II*) x1000 (TS)

Figure 3.13.6 Healthy disc tissue from assay water control



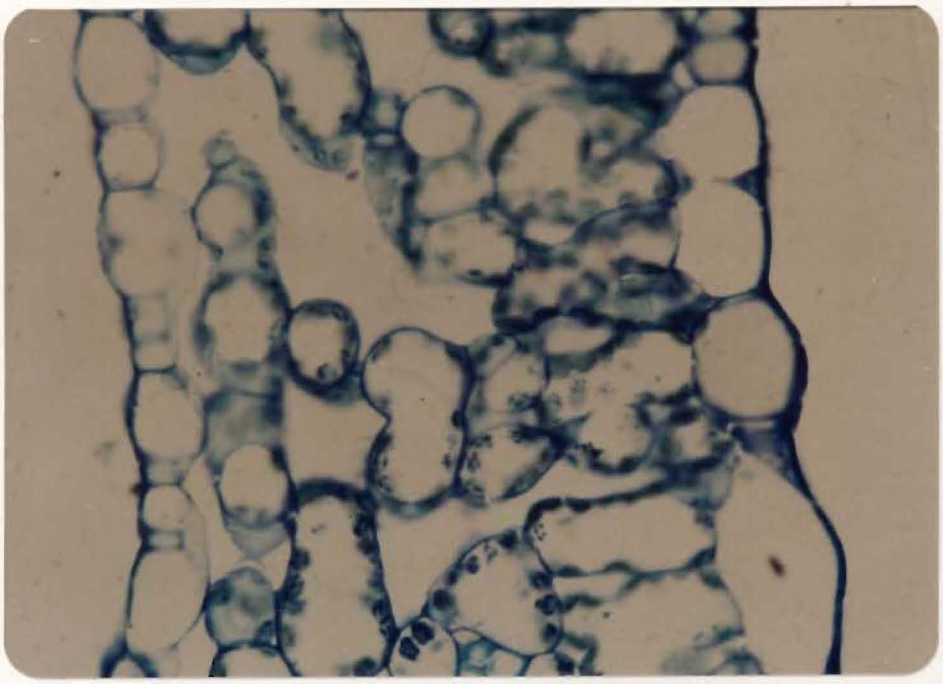
Choroplasts visible in normally distributed cytoplasm
(4 *u*m Spurrs/ Azur *II*) x400 (TS)

Figure 3.13.7 Chloroplasts in cells of water control tissue



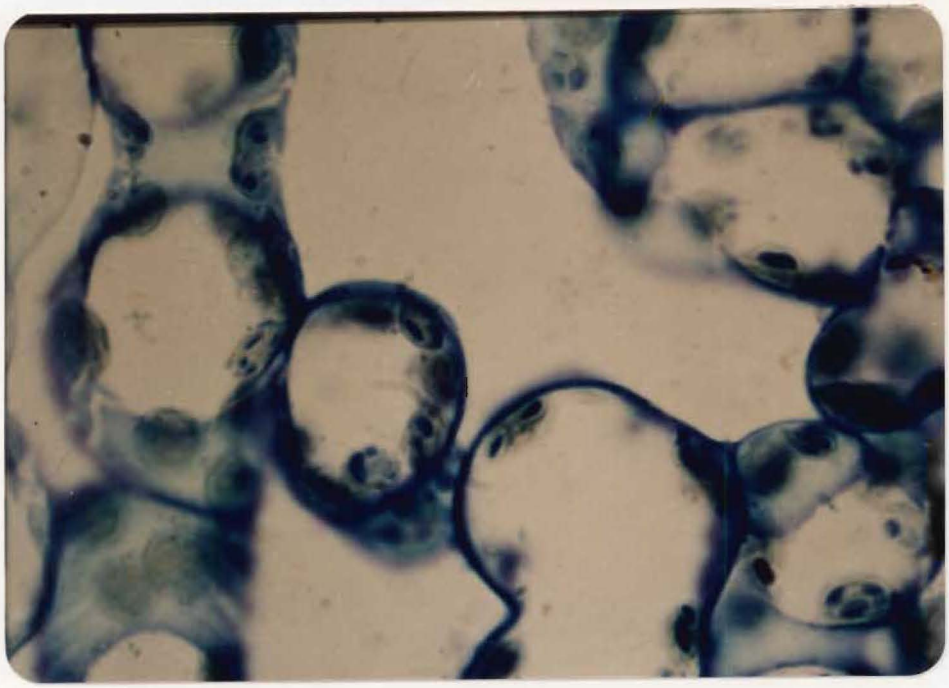
Starch grains evident in chloroplasts of mesophyll and
palisade cells (4 *u*m Spurrs/ Azur *II*) x1000 (TS)

Figure 3.13.8 Healthy disc tissue from assay solvent control



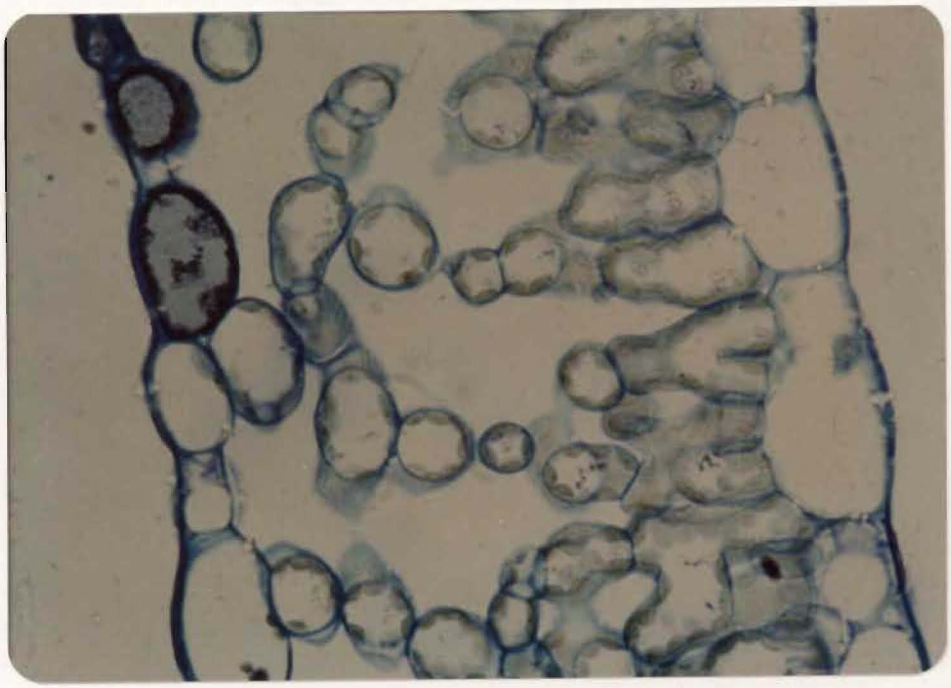
Choroplasts containing large starch grains visible in normally distributed cytoplasm (4 *u*m Spurrs/ Azur *II*) x400 (TS)

Figure 3.13.9 Chloroplasts in solvent control tissue cells



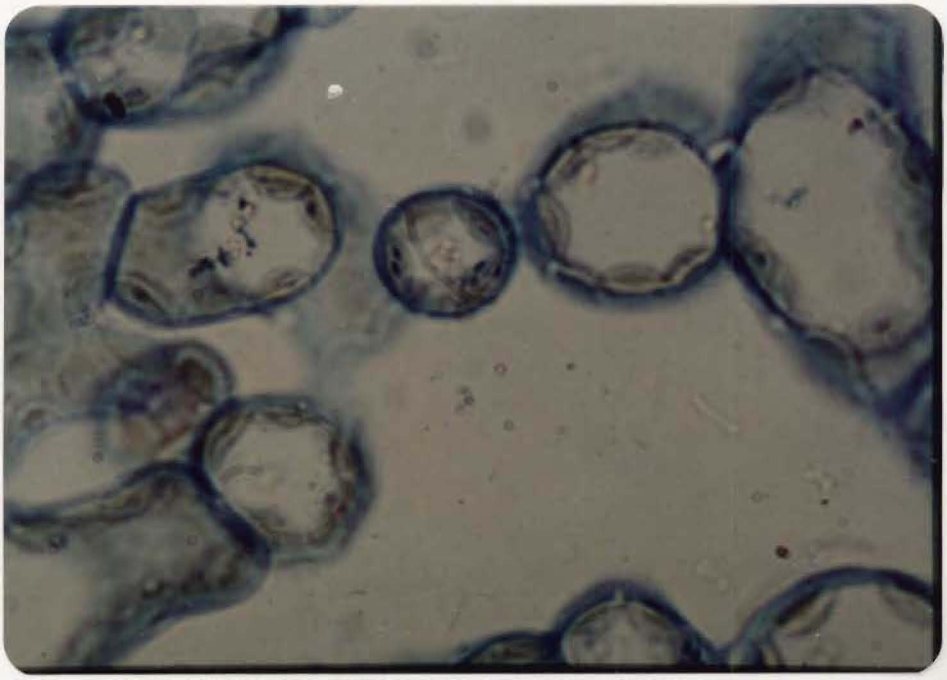
Presence of large starch grains in chloroplasts is the only visible effect of 5% (v/v) acetone (4 *u*m Spurrs/ Azur *II*) x1000 (TS)

Figure 3.13.10 Fresh leaf control



Choroplasts normally distributed in cytoplasm (4 *u*m Spurrs/ Azur *II*) x400 (TS)

Figure 3.13.11 Detail of fresh leaf cells



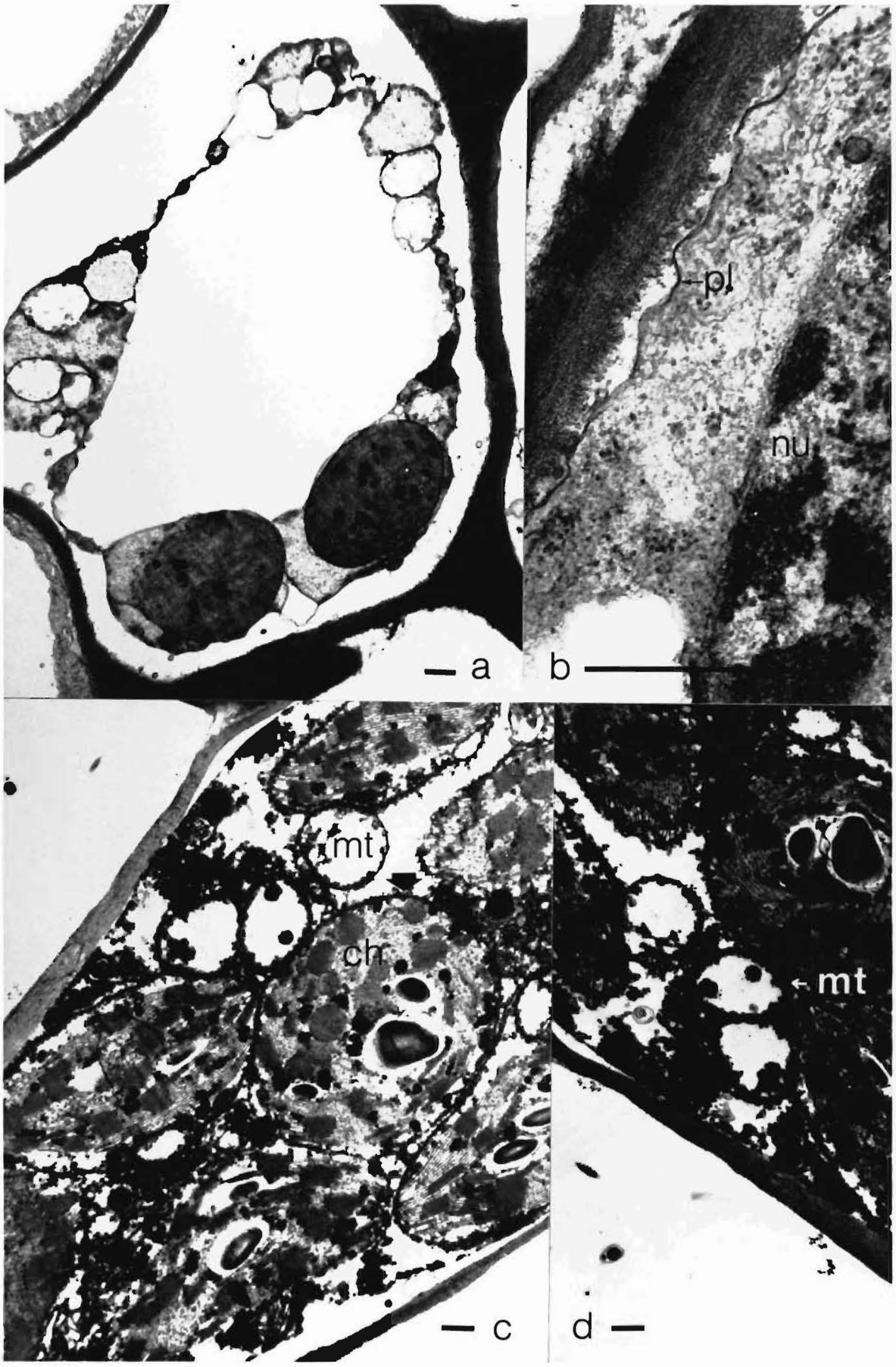
Chloroplasts normal size and distribution in mesophyll and palisade leaf cells (4 *u*m Spurrs/ Azur *II*) x1000 (TS)

facing page 130

Plate 13 Toxin damage to leaf cells

(a) Compression of cytoplasm and plasmolytic effect of toxin exposure on cell.
(b) Cell plasmalemma appears structurally intact and shows no visible signs of damage
(c) Limiting membranes of chloroplasts and mitochondria are indisinct and appear to have extraneous material associated with them (arrow)
(d) Mitochondria possess no visible internal structure

ch	chloroplast
mt	mitochondria
nu	nucleus
pl	plasmalemma

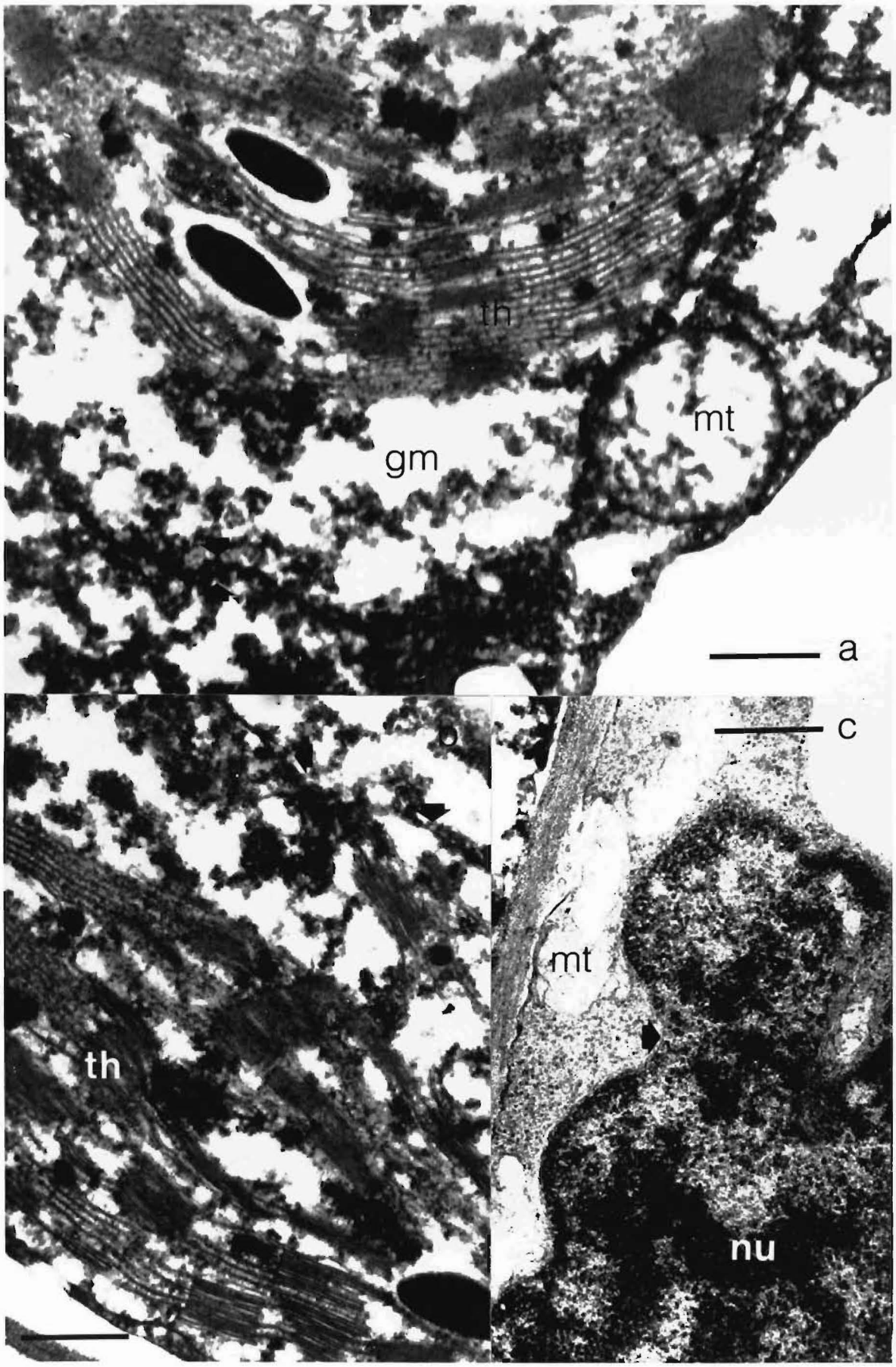

pl
nu
a
b
mt
ch
c
mt
d

facing page 131

Plate 14 Toxin damage to cell organelles

(a) Granal matrix and ground plasm appear 'granular' suggesting coagulation of matrix material. The chloroplast limiting membrane (arrows) is indisinct and unresolvable
(b) Some thylakoid stacks appear to be 'floating' inside the limiting membrane remains (arrows)
(c) The nuclear membrane also appears damaged as the typical double unit is indisinct (arrow). The tonoplast does not appear damaged at this stage

gm	granal matrix
mt	mitochondria
nu	nucleus
th	thylakoids


th
mt
gm
a
b
c
th
mt
nu

facing page 132

Plate 15 Dead leaf cells

(a) Typical appearance of dead cells from a leafspot. All organelle membranes appear to have disintergrated

(b) The plasmalemma still appears relatively intact

(c) Fungal hypha (TS) shows good fixation suggesting that observations on diseased tissue are not artifacts

pl plasmalemma


a
b
pl
c

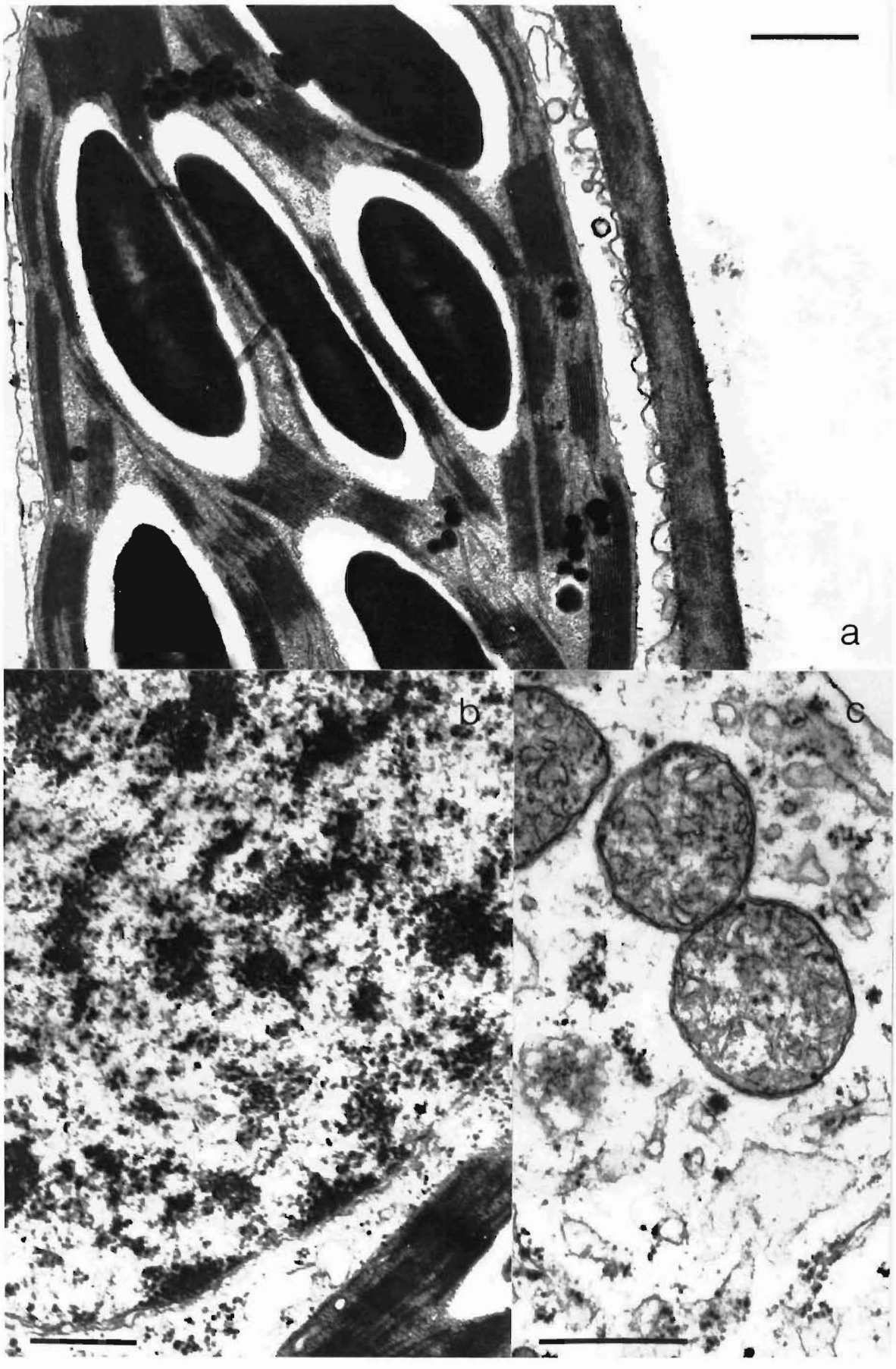
facing page 133

Plate 16 Ultrastructural effects of assay solvent on leaf cells

(a) Chloroplast membrane, plasmalemma, tonoplast, thylakoids and granal matrix all demostrate typical characteristics

(b) Nuclear membrane and nucleus

(c) Mitochondria with double limiting membrane and cristae


a
b
c

facing page 134

Plate 17 Normal leaf cell appearance

(a) Chloroplast and tonoplast

(b) Mitochondria and nucleus

(c) Plasmalemma and mitochondria

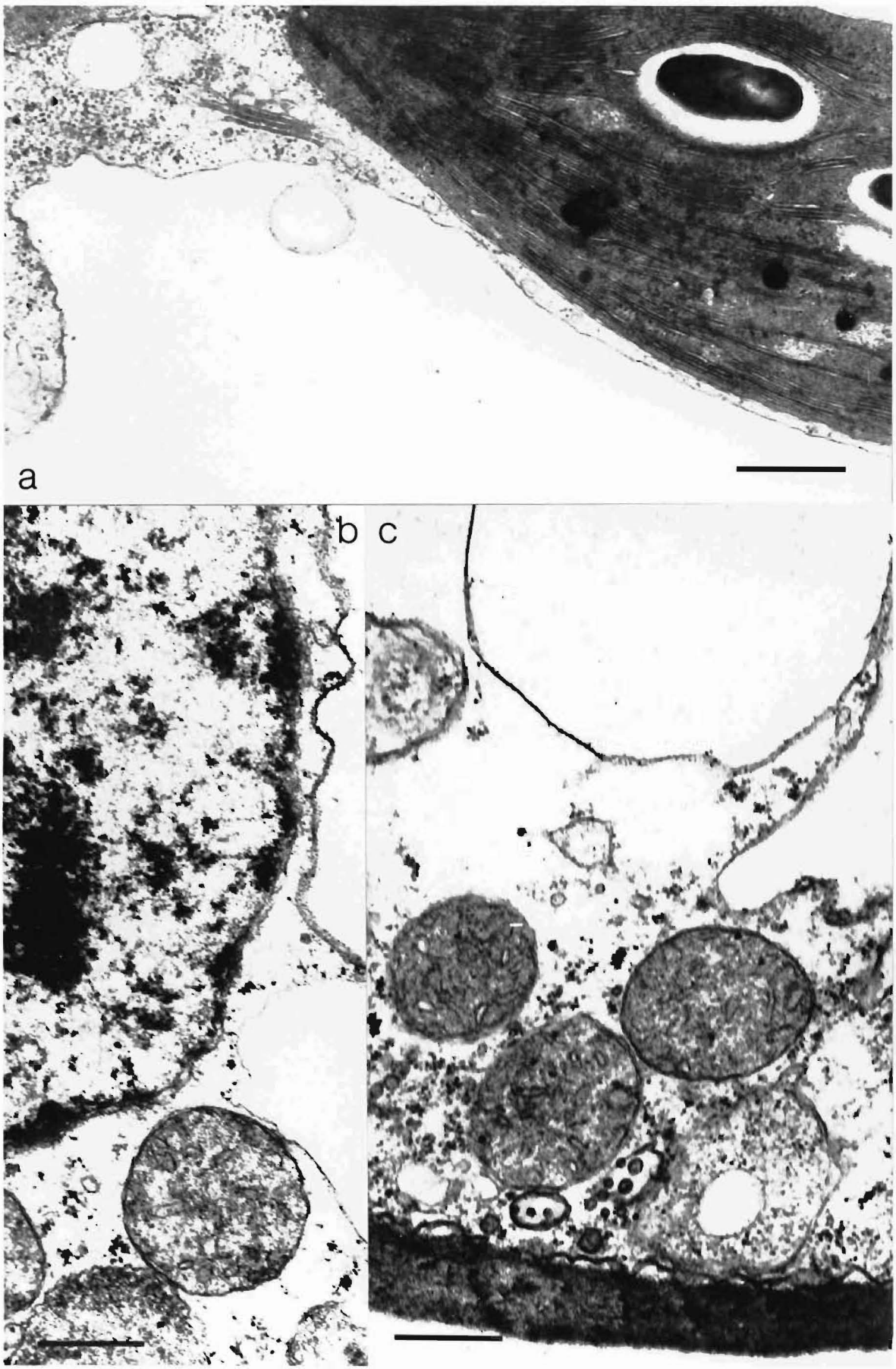

a
b
c

Figure 3.15.1 Toxin infrared spectrum (K Br)

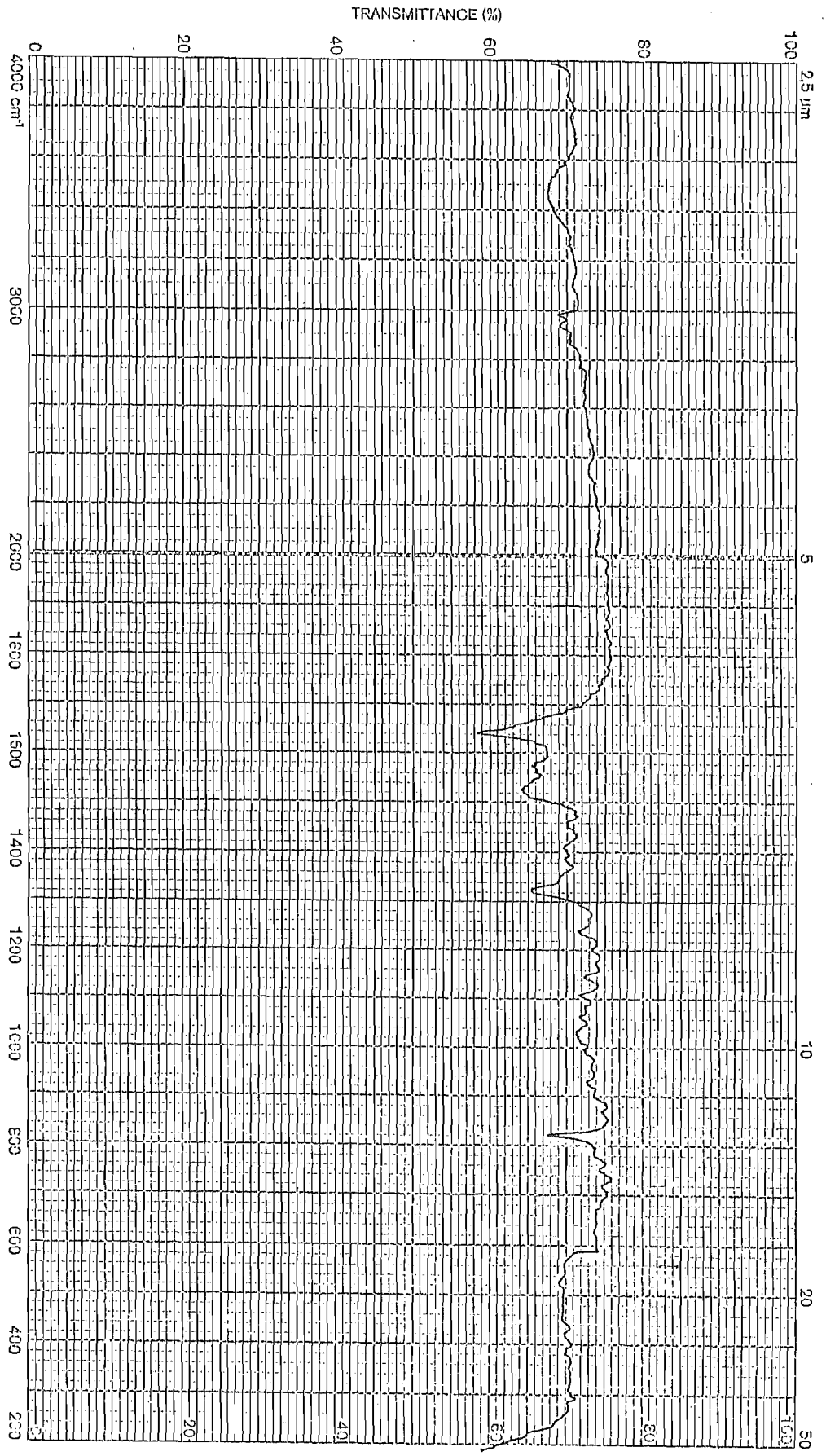


Figure 3.15.2 Toxin infrared spectrum (Nujol)

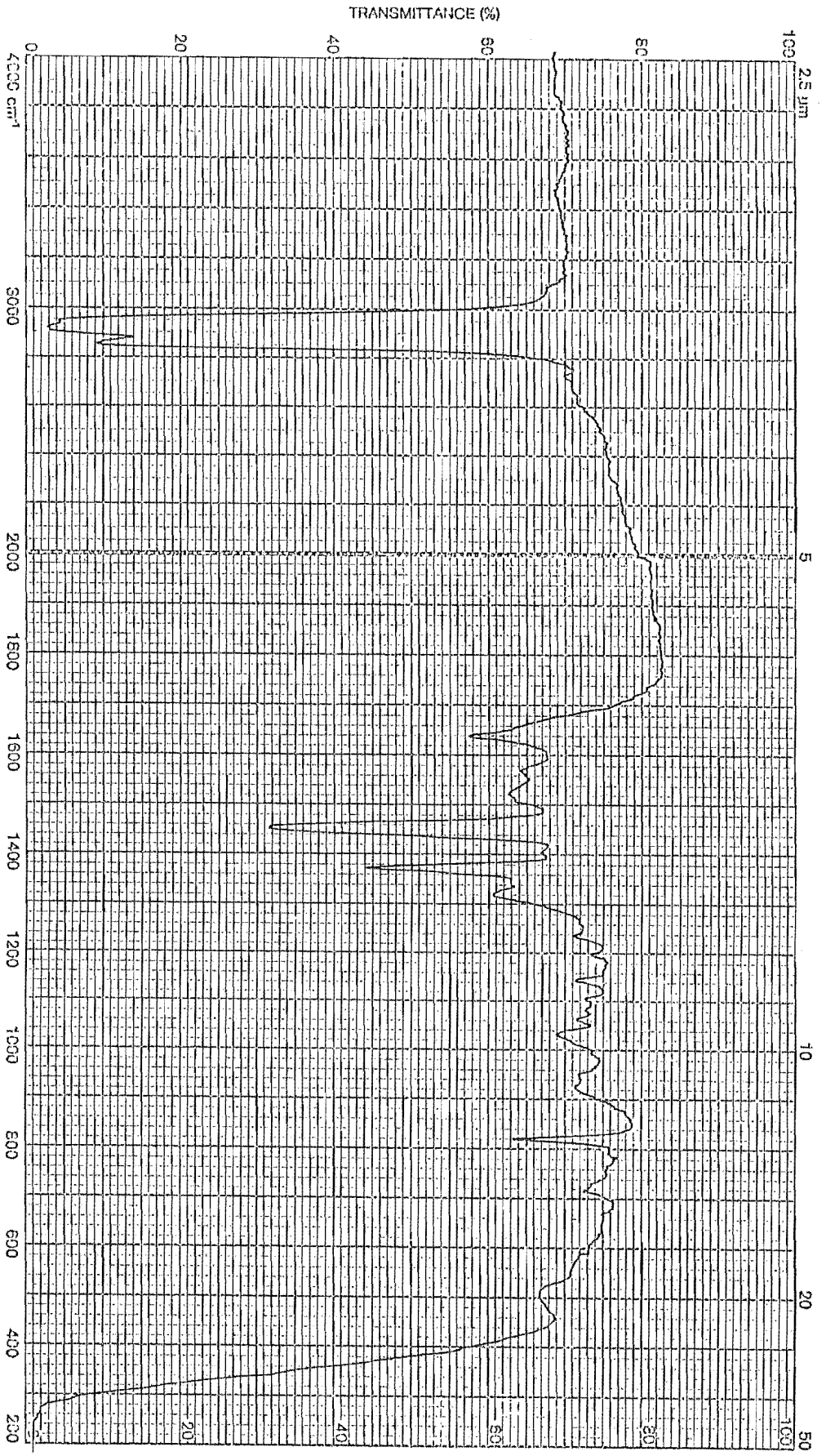


Figure 3.15.3 Toxin ^{1}H NMR spectrum

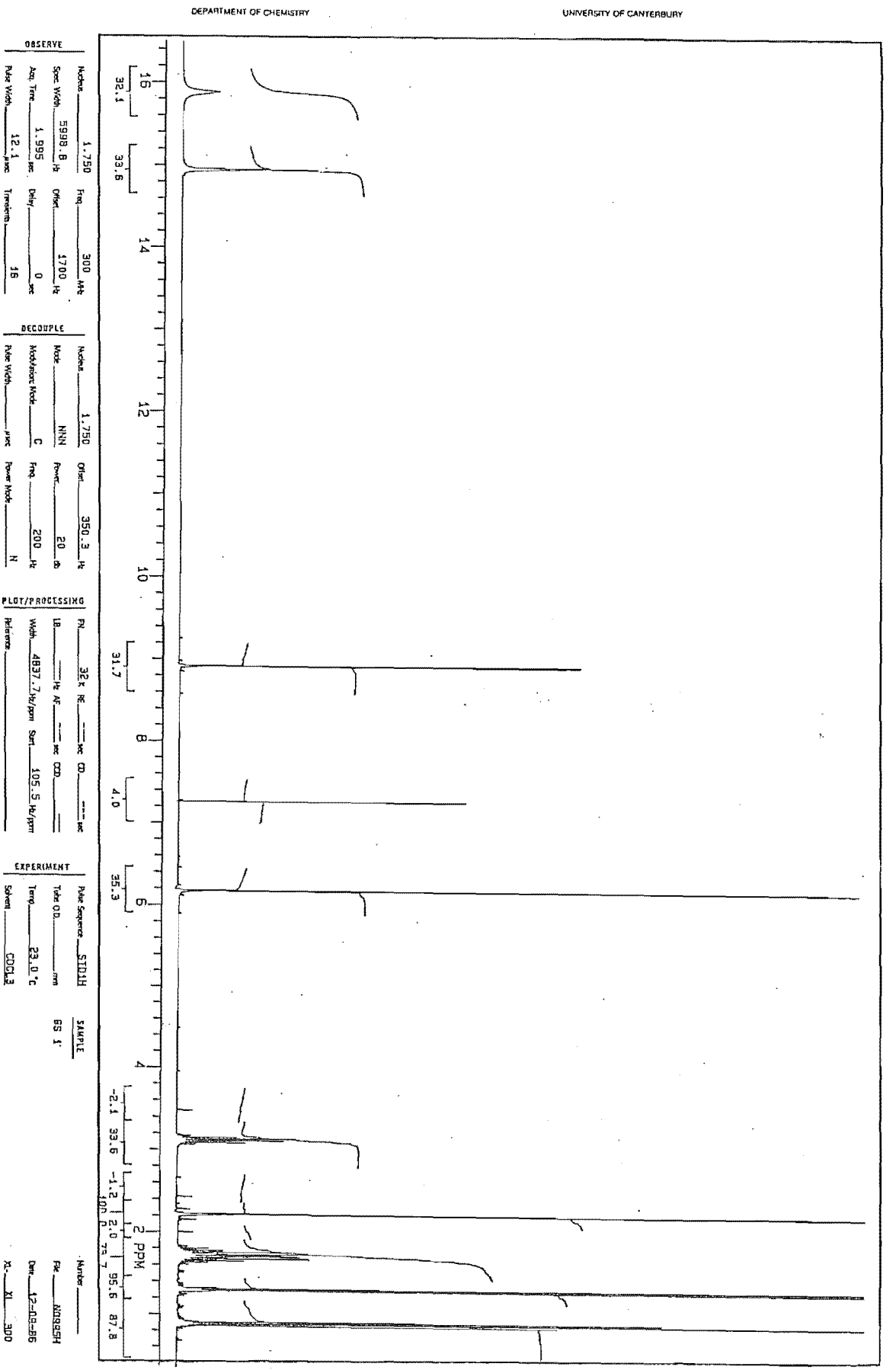


Figure 3.15.4 Toxin ^{1}H NMR spectrum

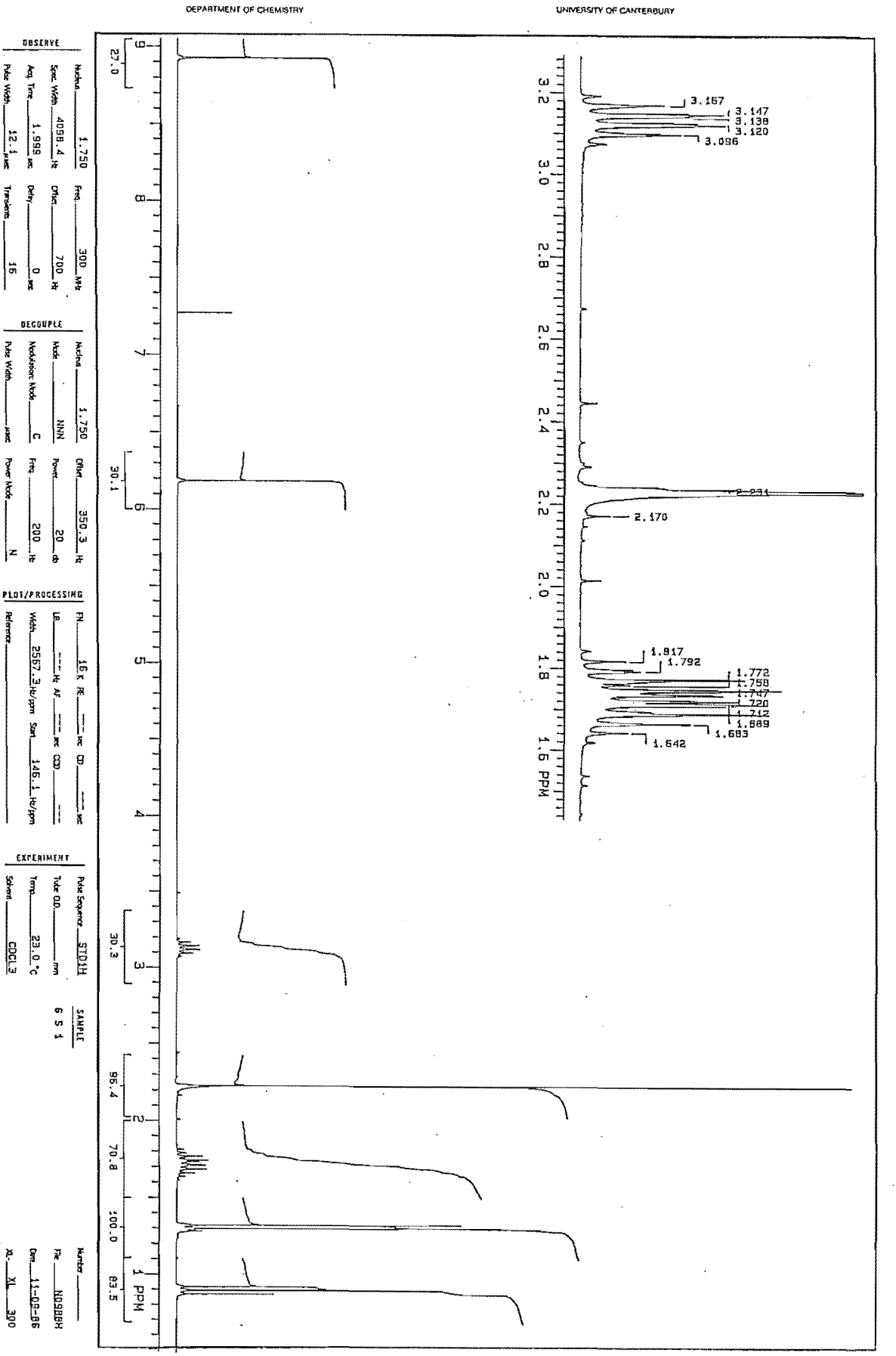


Table 3.15.1 Toxin ^{1}H NMR data

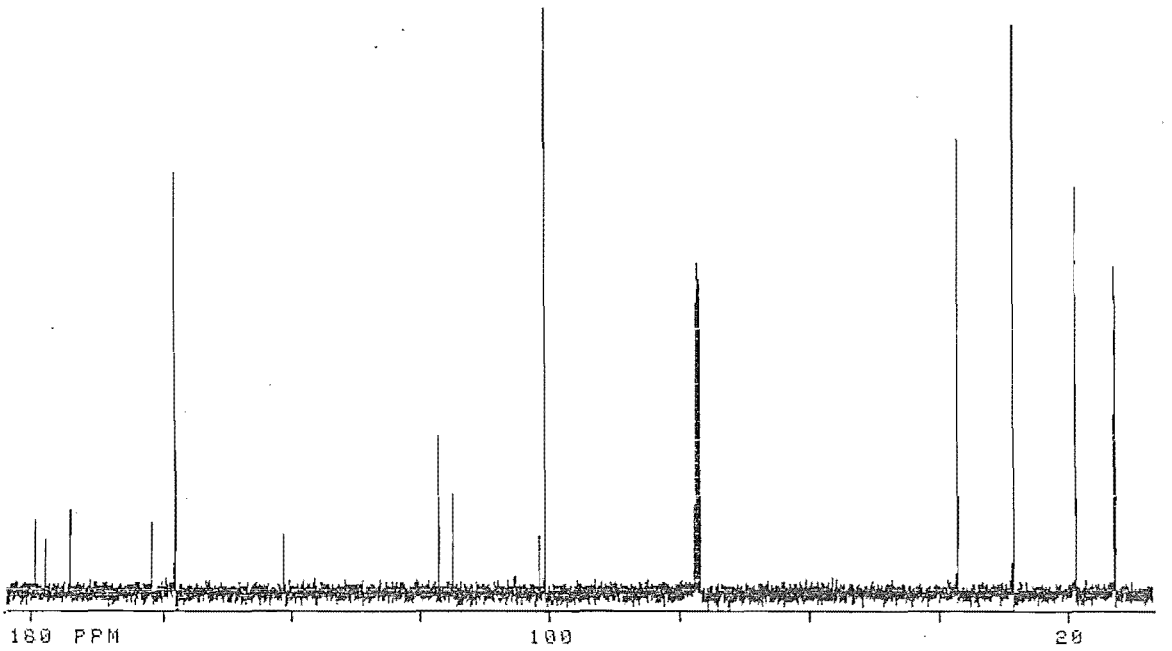
```
CC 1
EXP1  PULSE SEQUENCE: STD1H
DATE  12-09-88
SOLVENT  CDCL3
FILE     N0995H

OBSERVE PROTON
 FREQUENCY 299.93 MHZ
 SPECTRAL WIDTH 5999 HZ
 ACQ. TIME 1.995 SEC
 PULSE WIDTH 45 DEGREES
 TEMPERATURE 23.0 DEG. C.
 NO. REPETITIONS 16
 SPIN RATE 20 HZ
DATA PROCESSING
 FT SIZE 32K
DISPLAY
 WIDTH OF PLOT 16.13 PPM
 START OF PLOT 0.35 PPM
```

```
GS 1
SPECTRAL LINES FOR TH=    4.83
  RFL=   119.7  RFP=       0

INDEX  FREQ     PPM   INTENSITY
 01  4750.81  15.873   11.035
 02  4481.07  14.940   40.206
 03  2675.17   8.919  118.413
 04  2179.75   7.268   84.825
 05  1953.91   6.181  203.912
 06   949.66   3.166   17.979
 07   943.23   3.145   30.481
 08   935.17   3.118   31.319
 09   928.13   3.095   20.243
 10   921.67   3.073    5.223
 11   668.45   2.229  815.489
 12   649.53   2.166    5.629
 13   544.68   1.816    9.829
 14   539.15   1.798   10.874
 15   537.52   1.792   13.617
 16   531.30   1.771   33.764
 17   528.82   1.763   13.232
 18   523.24   1.745   39.017
 19   519.87   1.733   30.925
 20   513.23   1.711   37.224
 21   505.95   1.687   40.287
 22   498.59   1.662   20.609
 23   492.57   1.642    9.212
 24   390.81   1.303  335.103
 25   276.82   0.923  143.169
 26   269.53   0.899  317.479
 27   261.64   0.872  123.736
```

Figure 3.15.5 Toxin ^{13}C NMR spectrum



G S 1
SPECTRAL LINES FOR TH= 8.98
RFL= 277.5 RFP= 0

INDEX	FREQ	PPM	INTENSITY
01	13511.7	179.139	27.942
02	13398.9	177.643	20.447
03	13113.3	173.858	28.844
04	12183.9	161.535	24.397
05	11918.1	158.011	154.262
06	10647.2	141.161	11.137
07	10644.2	141.121	23.521
08	8836.4	117.154	53.887
09	8675.9	115.025	34.038
10	7645.9	101.370	19.747
11	7568.8	100.348	200.000
12	5832.1	77.323	108.725
13	5801.3	76.914	112.965
14	5768.7	76.482	110.496
15	2769.0	36.711	160.652
16	2099.3	27.833	195.022
17	1366.5	18.117	155.023
18	934.3	12.387	67.692
19	902.3	11.963	119.008

Figure 3.15.6 Toxin mass spectrum: EI

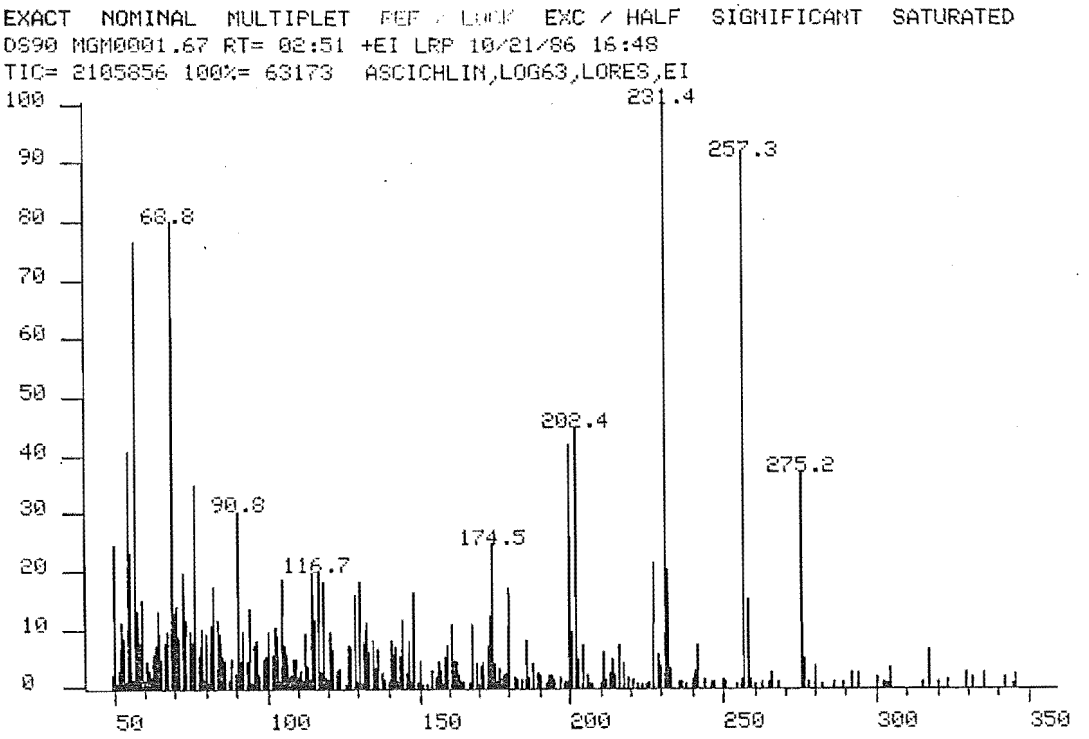


Figure 3.16.7 Toxin mass spectrum: CI

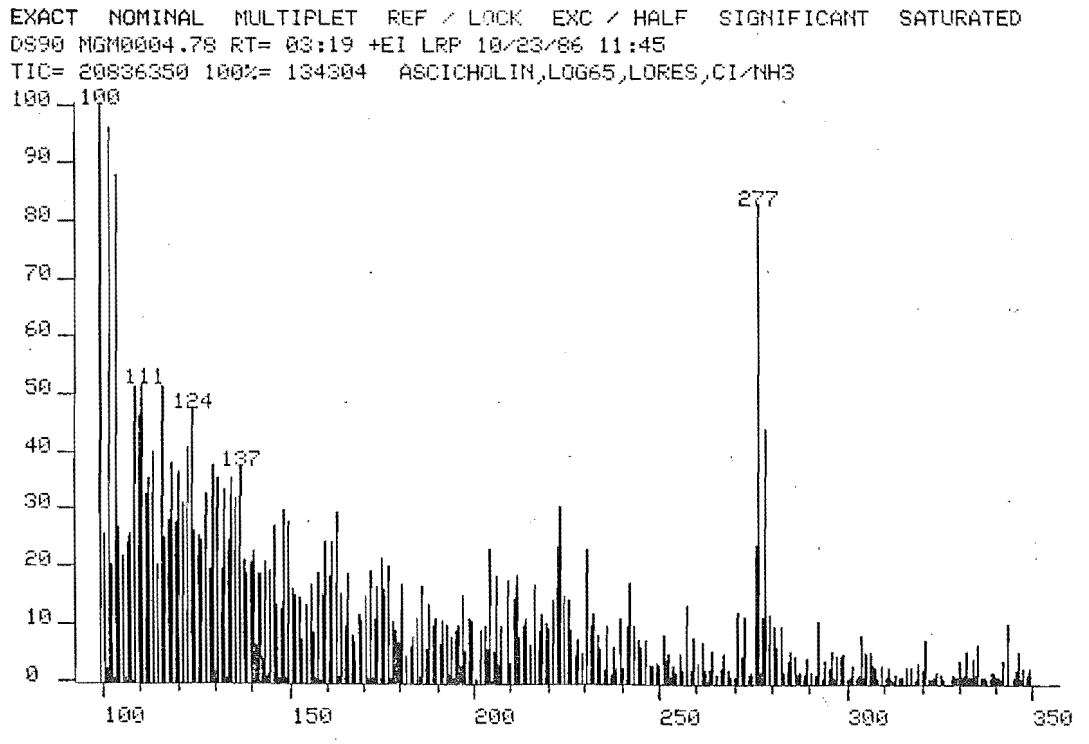
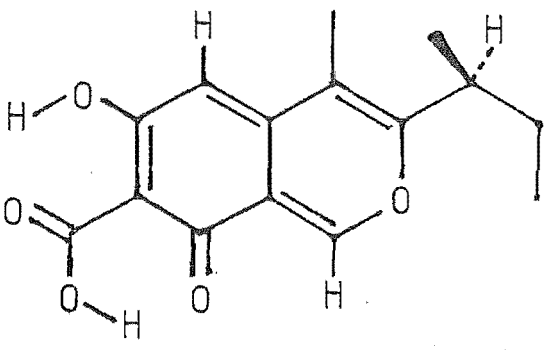


Figure 3.15.8 ortho-Ascochitine



3.15.3 Mass Spectroscopy

The theoretical molecular weight of 276.2 ($C_{15}H_{16}O_5$) was confirmed by electron impact (EI) (figure 3.15.6) and chemical ionisation (CI) (figure 3.15.7) spectra.

CHAPTER FOUR DISCUSSION

4.1 PATHOGEN TAXONOMY

The causal agent of leaf spot and wilt of large flowered *Clematis* hybrids and leaf spot of small flowered cultivars in New Zealand is identified as *Phoma clematidina* (Thum.) Boerema. Pathogen identification was based on pathogenicity towards *Clematis* cultivars and transmission electron microscopy of conidial ontogeny. Conidiogenesis was interpreted as phialidic (*sensu* Boerema and Bollen 1975) with the basal part of the conidial wall remaining as a collarette around the conidiogenetic locus of the conidiophore. This interpretation concurs with Boerema's (1979a) decision to transfer *Ascochyta clematidina* to the genus *Phoma*. The pathogen also produced mainly 1-celled spores *in vivo* and *in vitro*, typical of a *Phoma* species (Boerema and Bollen 1975).

Three strains were recognised from five New Zealand isolates, but could not be consistently related to type or herbarium specimens due to considerable variation existing between and among *P. clematidina* isolates. Gloyer (1915a, 1915b) had already noted the variability between isolates and felt any of the descriptions for *Ascochyta* (=*Phoma*) species found on clematis could apply, while isolates obtained by Ebben and Last (1966) showed 'appreciable morphological differences'. Assessment of a large set of criteria (appendix 7) indicated that isolate MT originally isolated from a leafspot on a 'Montana' leaf, was the most different isolate and was considered to represent one strain. Isolate EM appeared to represent an intermediate strain, between the MT strain and the strain represented by isolates LB, HD and RC. Other *Phoma* species have been identified on clematis including the ubiquitous saprophyte *P. nebulosa* (Boerema 1976) and *P. lingam* (Sutton 1980), but are associated with dead tissue.

Gloyer (1915a, 1915b) reported *A. clematidina* was not related to other common species of *Ascochyta*, and did not infect bean, pea, muskmelon, pumpkin, eggplant or elm. Boerema (1979a) noted that *A. clematidina* had been isolated from *Selaginella* (CBS culture 520.66). The relationship of New Zealand isolates to other *Ascochyta* and *Phoma* species especially to *A. pisi* and *A. fabae*, and the taxonomic significance of ascochitine production, to date only associated with *Ascochyta* species, are areas for further research.

The identity of the teleomorph of *P. clematidina*, despite numerous attempts to isolate or induce, was not established. The '*Phoma*-like' fungi have teleomorphs in several ascomycete genera. Most are represented in *Mycosphaerella* (26), *Leptosphaeria* (23), *Didymella* (12) and *Pleospora* (10) (Kendrick and Dicosmo 1979). Bos (1893) isolated from dead clematis tissue 'a fungus of the genus *Pleospora* or a cognate one', though this may have been *P. vitalbae* (syn. *P. clematidis*, anamorph *Hendersonia* species) (Webster and Lucas 1961). It is unlikely that the teleomorph is a *Pleospora* species as *P. clematidina* is sensitive to Benlate (ED_{50} 1.5 ug/ml). Bollen and Fuchs

(1970) found *Phoma betae* (= *Pleospora bjoerlingii*) was very resistant to 'Benlate', while all other *Ascochyta/ Phoma* species tested (with teleomorphs in *Didymella*, *Leptosphaeria* and *Mycosphaerella*) were sensitive (ED_{50} 1-5 ug/ml), and related benomyl resistance to teleomorph genera. *Leptosphaeria haematitis* (Rob. ex Desm.) Niessel recorded on clematis, (Ellis and Ellis 1985) is a possible teleomorph connection for *P. clematidina.*

4.2 PATHOLOGY

4.2.1 Disease Spread

Gloyer (1915a, 1915b) realised that "wilt" of clematis was not due to systemic invasion of the stem (cf *Fusarium*, *Verticillium*), but was the result of "stem girdling". '*Phoma*-like' vascular pathogens are not common but have been recorded in citrus (*Phoma tracheiphila* f. sp. *tracheiphila*), elm (*Plectophomella ulmi*) (cited Baker *et al* 1985) and chrysanthemum (*Phoma tracheiphila* f. sp. *chrysanthemi*) (Baker *et al* 1985). Leafspot and wilt are both symptoms of the disease, with the leafspot often progressing down the petiole into the stem, as 'the large-flowered types never shed their leaves cleanly' (Lloyd 1965). Gloyer's (1915a, 1915b) results suggest that *Phoma clematidina* is not a wound pathogen; plants sprayed with sterile water containing spores and kept under bell jars for two days developed water-soaked spots. Ebben and Last (1966) found that disease development on plants was 'irregular but increased by wounding'. In the present investigation *P. clematidina* was almost exclusively a wound pathogen in glasshouse trials. This may be related to the method of inoculation; in glasshouse trials spore suspensions were inoculated onto the adaxial leaf surface, while Gloyer (1915a, 1915b) noted that 'spores placed on the lower surface of the leaves produced more than those placed on the upper surface'. Infection through unwounded surfaces may be influenced by environmental conditions; Blok (1964) noticed more infection in high humidity situations, and in this investigation infection through unwounded plant surfaces occurred in the high humidity growth room trials.

4.2.2 Isolate Aggressiveness and Virulence

Infection trials of each isolate against the cultivar from which it was originally isolated indicated that all isolates were wound pathogens. The size of the lesion (virulence) was related to isolate aggressiveness. Once infection was successfully established fungal colonisation of surrounding tissue commenced. Analysis of contingency tables of isolate aggressiveness to cultivars indicated that except for isolate MT all isolates were equivalently pathogenic towards each cultivar.

4.2.3 Resistance

Ebben and Last (1966) suggested that 'differences in varietal susceptibility may be attributable to anatomical differences, e.g., in thickness of epidermal tissue, or age at which stems form a protective "bark"'. *Clematis* cultivars used in this study had similar amounts of bark. Bark thickness may be important in prevention of internodal rot, but plays little or no role in resistance to typical nodal rot as stem colonisation is via the petiole. Results of this study suggest that cultivar resistance to wilt and resistance to fungal colonisation of leaf tissue are based on two independent factors, although both factors can contribute to resistance. The ability of a cultivar to abscise or senesce infected leaves prevents fungal colonisation of the node from the infected leaf, while resistance to ascochitine lowers the rate of colonisation of the leaf.

Cultivars 'Lady Betty Balfour', 'Ernest Markham' and 'Rouge Cardinal' all exhibit similar susceptibility to fungal infection, and all are sensitive to toxin (either high or intermediate). None display leaf yellowing or abscission in response to infection and are classified as wilt susceptible, concurring with previous reports (Keay pers comm).

'Huldine' is very susceptible to infection and has an intermediate sensitive reaction to toxin suggesting it should be classified as a wilt susceptible cultivar. Yellowing of 'Huldine' leaves (presumably leaf senescence) prevents leafspot spread and subsequent petiole colonisation, conferring wilt resistance. Poor correlations between toxin sensitivity and susceptibility to fungal infection have been reported. Scheffer and Livingston (1980) found no correlation between toxin sensitivity and susceptibility to infection in three out of seventeen sugarcane clones tested, suggesting that toxin may determine pathogenicity to some clones but not to others. Senscence of plant tissue is usually correlated with high metabolic rate of the host-parasite complex at the infection centre (Farkas 1978). The large leafspots on 'Huldine' are the result of increased fungal growth possibly due to metabolic sinks arising at the infection centres leading to metabolite depletion and subsequent senscence of nearby tissues.

Cultivar 'Montana' displays both aspects of resistance. Leaf cells are resistant to toxin and leaf discs display resistance to fungal infection. Infected leaflets also resist fungal infection; those leaflets on which leafspots do develop abscise before the lesion reaches the petiole. Premature abscission of leaves is induced by a number of foliar pathogens (e.g. *Rhabdodine pseudotsuga* (needle cast of Douglas Fir), *Pseudopeziza medicaginis* (leaf spot of alfalfa)) (Cowling 1978), but appears to be regarded as a disease symptom rather than an expression of a resistance mechanism.

Resistance to ascochitine by 'Montana' leaf cells may be based on several mechanisms. Four basic mechanisms are the basis of microbial resistance to antibiotics; (1) alterations to permeability or transport system, (2) enzymatic inactivation, (3) alteration in target site by synthesis of a resistant or alternate site and (4) loss of the sensitive metabolic reaction (Arbuthnott 1984). Compensation for the inhibitory effect (e.g. by increased synthesis of inhibited enzyme) or circumvention of the blocked site

by operation of an alternate pathway are also possible, but seldom found, fungal resistance mechanisms to fungicides (Dekker 1984). While all these mechanisms are possibilities, biological reduction of ascochitine to dihydroascochitine (Oku and Nakanishi 1964a, Oku and Nakanishi 1966, Nakanishi and Oku 1969), as well as a low rate of ascochitine absorbance (approximately one fourth the rate of absorbance by sensitive fungi) appear to be characteristics of resistant fungi (Nakanishi and Oku 1969). While the reduction mechanism can be inhibited (sodium azide, cycloheximide, mercuric chloride) making resistant fungi toxin sensitive (Oku and Nakanishi 1966), in this study *Clematis montana* leaf cells could not be sensitized to toxin by cycloheximide, although the concentration of cycloheximide used was equivalent (by weight) to that which sensitized fungal tissue (Oku and Nakanishi 1966). Detoxification as the basis of 'Montana' resistance cannot be excluded as a mechanism by this result; further research should concentrate on the above areas to elucidate a resistance mechanism.

Further research is also necessary to elucidate the abscission/ senscence mechanism of resistance. Fungal infection stimulates the abscission mechanism while the leafspot edge is still 8 to 10 mm from the abscission zone. An interaction similar to that proposed for *A. pisi* and pea (van 'T Land *et al* 1975), in which ascochitine stimulates pisatin synthesis (Oku *et al* 1973, van 'T Land *et al* 1975) while pisatin inhibits ascochitine synthesis (van 'T Land *et al* 1975), may exist for *Clematis-Phoma clematidina* interactions.

4.3 FUNGICIDES

4.3.1. Protective Fungicides

The aromatics chlorothalonil ('Bravo') and dichlofluanid ('Euparen'), and the phthalimide captafol ('Difolatan') were the most effective fungicides in spore germination tests. 'Captan', the other phthalimide tested, also had a low MIC. The difference between the two phthalimides may be related to water solubility; captan is over twice as soluble as captafol (Worthing 1979). Of the dithiocarbamate fungicides zineb had the highest MIC and maneb the lowest. Zineb and maneb are the zinc and manganese complexes of nabam, while mancozeb is a co-ordinated complex of the zinc and manganese salts (Martin and Woodcock 1983). Nabam decomposes to the fungitoxic etem; manganese accelerating decomposition (Martin and Worthing 1976) and zinc increasing nabam stability (Martin and Woodcock 1983). These chemical properties account for the observed MIC values, the more stable zineb was less fungitoxic, and the activity of mancozeb lay between zineb and maneb. The systemic fungicides vinclozolin ('Ronilan'), prochloraz ('Sportak') and triforine ('Saprol') for which protective properties are claimed by the manufacturers had high *in vitro* MICs. Sulphur showed little fungitoxic activity, although 'sulphurous industrial fumes' appear to protect a *C. armandi* plant from wilt, at sub-phytotoxic concentrations (Lloyd 1977). Copper

oxychloride also showed poor activity despite Gloyer (1915a, 1915b) achieving some control of wilt with Bordeaux mixture.

4.3.2 Systemic Fungicides

The systemic fungicides showed a wide range of *in vitro* fungitoxicity, even within groups with the same mode of action. Methyl benzimidazol-2yl carbamate (MBC) is the active ingredient of the benzimidazole fungicides 'Bavistin' and 'Delsene'. Fungitoxic activity of 'Benlate' is attributed to hydrolysis of benomyl to MBC (Clemons and Sisler 1971, Bent 1979), while activity of thiophanate-methyl ('Topsin-M') results from facile conversion to MBC inside the plant (Martin and Woodcock 1983). Lack of "activation" probably accounts for thiophanates poor *in vitro* fungitoxicity. None of the MBC fungicides were fungicidal, as previously reported (e.g. Trinci 1984).

Ebben and Last (1966) suggested that the use of fungicides against *Clematis* mildew may indirectly reduce naturally occurring *Ascochyta*. 'Corbel', 'Sportak', 'Baycor', 'Calixin' and 'Bayleton' used to control a range of powdery mildew diseases, demonstrated variable activity against *P. clematidina*. The most effective, 'Corbel' and 'Sportak', were both fungistatic and fungicidal, while 'Baycor', 'Bayleton' and 'Calixin' did not affect fungal growth.

The ergosterol biosynthesis inhibiting (EBI) fungicides constitute the largest and most important group of systemic compounds yet developed for control of fungal diseases of plants and animals. They control a wide spectrum of pathogens often exhibiting very high antifungal activity (Sisler and Ragsdale 1984). Of the triazole subgroup only propiconazole ('Tilt') possessed fungistatic and fungicidal activity. It would be interesting to test etaconazole ('Sonax', CGA 64251) for activity as it only differs from propiconazol in having a shorter side chain (C_2H_5 against C_3H_7). The most effective fungicide tested was the EBI fungicide fenpropimorph ('Corbel'). 'Corbel' displays good *in vivo* activity against *Puccinia* species, *Erysiphe graminis*, and *Rhynchosporium secalis* (Maag 1982). The morphilines are presumed to act by inhibiting sterol $\Delta^{14(15)}$ reduction or Δ^{8} to Δ^{7} isomerisation (Sisler and Ragsdale 1984). Tridemorph ('Calixin)', the other morphiline tested did not exhibit any *in vitro* activity. Unless tridemorph requires "activation" (c.f. thiophanate-M), then these fungicides show remarkable *in vitro* selectivity. It would be interesting to extend these trials to another morphiline, dodemorph ('Meltatox', BAS 238F), in this respect.

The other fungicides tested demonstrated poor fungitoxicity against *P. clematidina*. Carboximides ('Calirus') are more or less selective towards basidiomycetes (Kuhn 1984), dicarboximides are used mainly to control *Botrytis* and related genera (Leroux and Fritz 1984), while kasugamycin was utilised to control the rice-blast fungus, *Pyricularia oryzae* (Martin and Woodcock 1983).

4.3.3 Fungicide Interactions

Theoretical models and practical examples suggest that 'there is a significant delay of resistance build-up when mixtures of fungicides with different modes of action are used' (Gisi *et al* 1985). Chlorothalonil ('Bravo') is synergistic to the EBI fungicide 'Fenarimol' (a pyrimidin-5-yl methanol) (de Waard and van Nistelrooy 1982), and is added (333 g/l) to 'Corbel' (200 g/l) to extend the spectrum of activity of 'Corbel' (marketed as 'Corbel-Star') (Maag 1982). In the present *in vivo* investigation chlorothalonil either did not interact with fenpropimorph ('Corbel') or at one fenpropimorph concentration acted as an antagonist. The reasons for this are unclear but may be due to complex formation, partioning of fungicide into undissolved residues of the antagonist or chlorothalonil affecting cellular permeability and preventing fenpropimorph reaching its site of action (de Waard and van Nistelrooy 1982).

4.3.4 Fungicides in the Glasshouse

The extrapolation of *in vitro* results to the *in vivo* situation must be made with caution. *In vitro* antifungal activity of benomyl was shown by Bollen and Fuchs (1970) to be almost completely correlated with field effectiveness. Predictions of field performance of triforine could not, however, be based on *in vitro* activity (Drandarevski and Fuchs 1973). As previously noted Gloyer (1915a, 1915b) found that a sulphur mixture or Bordeaux mixture sprayed on cuttings or bedded plants controlled the disease, although neither sulphur nor copper oxychloride were effective *in vitro* fungicides in this study.

The fungicide mixture (fenpropimorph/ chlorothalonil) acted to delay leafspot initiation and hence decrease leafspot size with respect to time. This mixture acted best as a protectant fungicide, possibly because 'Corbel' has a short half-life in *Clematis* (approximately 5 days in wheat (Maag 1982)), or was not systemic in *Clematis* tissue, remaining on the surface. A single 'Corbel' treatment is considered 'sufficient to keep barley, wheat, rye or oats practically disease-free during the growth stages which are most important for yield production'(Maag 1982), although to control *Clematis* wilt in the glasshouse a more regular (weekly) spray period would probably give better disease control.

4.4 TOXIN CHEMISTRY

4.4.1 Identification

The toxin isolated from *P. clematidina* culture filtrate and leaf lesions was identified as ascochitine ($C_{15}H_{16}O_5$) by ^1H NMR spectroscopy. Identification was confirmed by ^{13}C NMR spectroscopy and mass spectroscopy. Ascochitine was originally assigned as a *para*-quinone-methide by Iwai and Mishima (1965), but after analysis of long-range coupling constants Colombo *et al* (1980) assigned ascochitine as the *ortho*-

quinone-methide, although the structurally and chemically related toxin citrinin, is assigned as the *para*-quinone-methide (cited Colombo *et al* 1980). (±)- ascochitine was chemically synthesized in 1966 by Galbraith and Whalley. The present ascochitine preparation appeared to be a mixture of the two tautomers (Munro pers comm). While biochemical precursor studies show that ascochitine is derived from a hexaketide chain and three C_1 units via the polyketide route (Colombo *et al* 1980), enzymes of secondary metabolism are noted as having decreased sensitivity to substitution pattern variations, meaning in many cases all the metabolic traffic does not proceed by the same route from starting material to end product (Haslam 1985). If different chemical operations are carried out in a different order, this can lead to isomer production. This would explain the results obtained but raises the issue of which isomer is biologically active. Future assay research should establish the activity of each isomer and the equilibrium between them.

4.4.2 Production, Isolation and Purification of Ascochitine

Techniques for the production and isolation of microbial toxins are similar to those for other products of microbial metabolism (e.g. vitamins, antibiotics). Ascochitine has been isolated from culture broth of *Ascochyta pisi* (Bertini 1956, Lepoivre 1981, 1982a), *A. fabae* (Oku and Nakanishi 1963) and *Ascochytula obiones* (cited Turner and Aldridge 1983), while *Mycosphaerella pinodes* (=*Ascochyta pinodes*) produces ascochitine *in vivo* but not *in vitro* (Lepoivre 1982b). Ascochitine (and its reduction product dihydroascochitine) are quantitatively extractable from acidic aqueous solution with chloroform (Nakanishi and Oku 1969). Ascochitine isolated from culture broth of *P. clematidina* isolates and purified by Lepoivre's (1982a) method was not totally pure. Lepoivre's method utilises Hald and Kroghs' (1975) method for extracting citrinin, and gives a pure product when isolating ascochitine from *A. pisi* culture broth (Lepoivre 1982a). Demonstration of product purity was essential before toxin identification by NMR spectroscopy. For this reason a purification method based on methanol solubility differences was developed. The orange colour of a 24h methanolic toxin solution and contaminant solution, as well as toxin/ contaminant co-isolation suggests the contaminant is chemically similar to the toxin, most probably either a degradation product or dihydroascochitine. No attempt was made to identify this product. Citrinin in solution is recorded as beginning to decompose at 60-70^{o}C and photodecomposes in UV or ambient light (Neely *et al* 1972).

Solubility of ascochitine isolated and purified in the present study did not agree with published results. Lepoivre (1982a) utilised 100 ug/ml ascochitine in 2% ethanol for his bioassay, while in this study maximum toxin solubility was 25 ug/ml in 5% acetone. This result may be related to the different definitions of purity between the two studies.

4.4.3 HPLC Analysis

Pure ascochitine (as defined by two dimensional TLC) was analysed by reverse phase HPLC to determine preparation purity. Protocols exist for HPLC analysis of food, foodstuffs and biological fluids (bile, urine) for the nephrotoxic citrinin either alone (e.g. Phillips *et al* 1980) or in combination with other 'mycotoxins' (Lepom 1986). No reported procedures exist for HPLC analysis of ascochitine, thus development of a suitable HPLC system was based on reported procedures developed for citrinin.

Citrinin is chromatographed in methanol or acetonitrile with an acid phase to suppress ionisation and subsequent peak tailing (Marti *et al* 1978, Phillips *et al* 1980). Low ascochitine retention and poor peak resolution with methanol based elutents suggested methanol was too strong a solvent (E° too high) (Synder and Kirkland 1974). Additions of ethyl acetate (unpublished data) (Phillips *et al* 1986), or use of TFA (Munro pers comm) as the acid phase did not improve either parameter.

Ascochitine chromatographed in acetonitrile: phosphoric acid showed better resolution, though peak resolution was again adversely affected by decreasing solvent concentration. Retention time was again low, but the ascochitine peak was separated from the solvent peak (void volume). While the final elutant system does not satisfy HPLC criteria ($0.8<t'^{*}$, $2<k'<6^{*}$ (Phillips *et al* 1980)) the curve shape compares favourably with published citrinin curves (Marti *et al* 1978, Phillips *et al* 1986, Lepom 1986), and demonstrates purity of the ascochitine preparation.

Improvements to liquid chromatography of ascochitine may require the use of silica gel columns eluted with water saturated chloroform: glacial acetic acid (Hunt *et al* 1978 for ochratoxin A and aflatoxins B_1, B_2, G_1, G_2 and M_1), rather than refinements of reverse phase systems (e.g. acetone: acid elutants).

* (see appendix 9 for definitions)

4.5 ASCOCHITINE BIOASSAY

4.5.1 Criteria for a Toxin

As already noted, no single criterion is adequate to establish causal roles for toxins in plant disease. In 1980, Yoder wrote that 'most criteria that have been used to evaluate the pathological significance of toxins (e.g. symptom production by toxin, presence of toxin in the plant, kinetics of toxin production during disease development, correlation of toxin biosynthetic rate with virulence) are logical but in practice are inconclusive as primary lines of evidence'. 'Each potential toxin must be considered on its own merits' (Scheffer and Briggs 1981), although results obtained with model toxin systems must be interpreted with caution, as it unlikely that any single metabolite will be found to be solely responsible for all aspects of a plant disease (Wheeler 1978). In

this study a combination of toxin criteria and assay criteria established ascochitine as a causal factor in disease expression.

4.5.2 Assessment of Toxin Criteria

In the present investigation there was a relationship between *in vitro* ascochitine production and isolate virulence. The qualitative correlation of virulence with the *in vitro* production of toxin provides a 'very persuasive line of evidence' though 'quantitative assessments are generally inconclusive' (Yoder 1980). *In vitro* production is influenced by media composition and physical environment and may not be related to natural production, 'as high producers in one set of culture conditions may be low in another' (Yoder 1981).

Ascochitine was isolated from *Clematis* leaf lesions, and has also been isolated from pea leaves infected by *A. pisi* and *M. pinodes* by Lepoivre (1982b). Presence of toxin in the infected plant is dismissed by Yoder (1980) as 'of little value'. He considers the 'presence in diseased tissue, especially in visibly affected areas, only indicates that when the pathogen grows toxin is produced; this does not indicate that toxin is causing disease rather that resulting from it'.

Blackening of leaf discs exposed to ascochitine, similar to tissue blackening in natural leaf lesions, was related to toxin concentration and exposure time. Yoder (1980) regards a "typical disease symptom" as 'one of the least reliable criteria for evaluating a possible role for a toxin in disease'. Durbin (1972) and others (e.g. Daly 1976, 1981) note that plants have a limited number of ways of responding to disturbance. The visible symptom may be caused by a factor which may or may not be involved in the disease: 'for example, it is not uncommon to confuse symptoms of herbicide injury with those of infectious disease' (Yoder 1980).

'In the physiological complexity of the host-parasite interaction it is difficult to distinguish causes from effects' (Yoder 1980). Combinations of toxin criteria increase confidence that the suspected toxin has an active role in disease expression, with results from the present quantitative assay and ultrastructural investigation supporting this conclusion.

4.5.3 Choice of Assay

'A suitable assay is basic to further work and characterisation of any natural product...' (Scheffer 1976). Numerous assay systems are described in the literature including seedling root growth, dark CO_2-fixation, mitochondrial respiration, electrolyte leakage, protoplast death, the uptake of test solutions by the open vessels of shoot-cuttings or wounds in the leaf laminae, and the inhibition of various enzyme systems (e.g. succinate, NADH). The purpose for which the assay is developed, 'determines the characteristics of the assay that are most appropriate. For example, high toxin sensitivity is required for monitoring toxin purification whereas the priority is on efficiency when screening for disease resistance' (Yoder 1981). The five main purposes

for which assays are developed are (1) to detect new toxins; (2) monitor purification; (3) determine mechanism of action; (4) determine role in disease; and (5) screen for disease resistance (Yoder 1981).

At present 'the most widely employed test for activity of pathotoxins is that involving induction of endogenous ion leakage' (Daly 1981). Toxin induced changes in membranes and release of electrolytes and other cellular material have been the basis of many assays (e.g. Luke *et al* 1969), but routine assays based on an increase in permeability 'have not been used for non-host specific toxins in contrast to host-specific toxins' (Rudolph 1976).

The permeability assay has been utilised since, at least, 1939 when Thatcher used a plasmolytic method to compare the effect of various fungi on the the permeability and osmotic pressure of host cells (Page 1972). The use of the conductivity bridge to quantify electrolytic leakage appears to have its origins with the work of Wheeler and Black on the effect of victorin on oat tissue (e.g. Wheeler and Black 1963).

Electrolytic leakage assays have been developed for both 'host-specific' and 'host-nonspecific' toxin studies (i.e. Scheffer and Livingston 1980, Lepoivre 1982a). Daly (1981) stated that 'the relative ease by which this assay can be carried out is perhaps unfortunate'. In the past, the procedure tended to focus attention on permeability of the plasmalemma, perhaps to the exclusion of other considerations'. The equilibrium of ions between the cell and its environment (or between organelles of a cell) is the difference between the influx and the efflux of those ions. The attention given to the efflux of ions from a cell (or tissue) has 'tended to minimize other approaches to the study of flux rates' (Daly 1981). Daly further notes that 'there is a general belief that ion leakage is detrimental to cells and therefore it is a significant sign of toxicity that requires further investigation'; Yoder (1981) states that 'cell death and electrolyte leakage can be independent events'.

'There can be no standard recommendations regarding assays because of the diverse tasks assigned to them' but 'credible work with toxins depends on appropriate selection and use of assays' (Yoder 1981). The toxin produced by isolates of *P. clematidina* had several chemical characteristics in common with citrinin and ascochitine. Permeability studies with these 'host-nonspecific' toxins have indicated that these quinonemethides act against the integrity of the cell membrane (Betina and Barathova 1968, Lepoivre 1981, 1982a). The choice of an electrolyte leakage assay for quantification of the *Clematis* cultivar response was largely influenced by the literature. The most reliable bioassays have been based on distinctive toxin effects (Scheffer and Briggs 1981); the quantifiable response in the present investigation suggests that an electrolyte leakage assay was appropriate.

4.5.4 Assay Parameters

The assay was developed with cultivars 'Lady Betty Balfour' considered most susceptible to wilt and 'Montana' the most resistant to wilt (Keay pers comm). A

response was induced in 'Lady Betty Balfour' leaf discs by 1 ug/ml ascochitine solution, but the assay lacked sensitivity to statistically differentiate this response from the solvent response. Lack of precision has been found in other leakage assays, Wheeler and Black (1963) noting differences of 10% between replicates. Although this assay lacked the sensitivity to differentiate responses below 5 ug/ml, it was sufficiently sensitive to distinguish 'resistant' tissue at this toxin concentration. The assay also indicated that at concentrations above 5 ug/ml 'resistant' tissue became sensitive and leaked. This result is in contrast to Lepoivre's (1982a) results with ascochitine where resistant and sensitive pea leaf tissue leaked equivalent amounts of electrolytes until a toxin concentration of 25 ug/ml when sensitive tissues leaked markedly more than resistant tissues; this relationship was maintained with increasing ascochitine concentrations.

'Investigation of the dose-response relationship is essential for most purposes to which bioassays are put and cannot be overemphasized because it is frequently ignored' (Yoder 1981). The response time of this assay was critical to achieving quantitative results. The decision to compare the bathing solution conductivities at 20h was based on both the shape of the time response curves and statistical analysis of results. By replotting this data a sigmoidal dose response curve was found. It was important to determine the dose response curve shape to elucidate the minimum and saturation doses. The plateau of this curve was the assay toxin saturation concentration, which was used in toxin site of action studies. Straightening the sigmoid curve with a $\log_{10}$ transformation and correlating to a regression line confirmed data fit to the curve.

Leaf discs of the resistant cultivar weigh more than leaf discs from sensitive cultivars. Damann *et al* (1974) found that large leaf samples lose electrolytes faster than smaller samples, a phenomenon apparent in this investigation. The present studies showed that tissue weight was an important parameter of the assay, rather than number of leaf discs. Most authors comment on the weight of leaf discs or tissue used, but few appear to have considered that leaf discs from different cultivars may have different weights thus affecting the sensitivity of their assays. For instance, Scheffer and Livingston (1980), who used 1cm leaf discs to assay sugarcane clones for sensitivity to *Helminthosporium sacchari* toxin noted 'each vial contained eight discs (=0.1g) in 2ml of toxin solution or water', but did not mention cultivar disc weights. Correcting each replicate by a scaling factor related to tissue weight before statistical analysis may improve assay sensitivity. The large cell constant of the conductivity probe also decreased assay sensitivity. Increased sensitivity would permit a more critical evaluation of the minimum toxin concentration necessary to induce endogenous ion leakage and allow for possible redefinition of this value.

While the solvent had a measurable effect on leaf tissue, inducing some endogenous ion leakage, the evidence (curve shape, appearance, light and transmission electron microscopy) suggests this leakage was not damaging, and supports the decision to to subtract solvent response from overall response to give leakage attributable to toxin activity (net solution conductivity).

4.6 SITE AND MODE OF ACTION OF ASCOCHITINE

The molecular site of action of a toxin is determined from bioassay results. Usually the site is not known so a mode of action is inferred from bioassays involving whole tissues, cells or organelles (Yoder 1981). The "classical" site of action of toxins inducing electrolyte leakage is the plasmalemma; the "classical" mode of action is to alter membrane permeability.

The mode of action of ascochitine inferred from electrolyte leakage studies is an alteration to plasmalemma permeability. The site of action was investigated using the transmission electron microscope, as Daly (1981) recognised 'potentially very useful tools for identifying cellular toxin sites are cellular symptoms recorded with high resolution light or electron microscopy'. Few ultrastructural studies have been published despite the large number of described toxins (Hanchey 1981).

Chloroplasts and mitochondria showed marked effects after ascochitine treatment, while the plasmalemma and tonoplast did not. The plasmalemma appears intact even after cell death, suggesting it retains structural integrity long after loss of functional (e.g. selective permeability) properties. In bioassay leaf cells, mitochondria show the greatest response to ascochitine; in leafspot cells this situation is reversed. This probably indicates that the bioassay parameters do not accurately reflect the natural disease situation (i.e. leaf cell exposure to isolated toxin for a short time period).

Fixation artifacts are of concern in any ultrastructural study, and 'may increase in frequency in already weakened membrane systems' (Hanchey 1981). Diseased or toxin damaged tissue appears well fixed, based on appraisal of fixation of associated healthy tissue beyond the leafspot edge, hyphae ramifying through the leafspot and solvent control tissue. Cell organelles revealed analogous responses to toxin treatment though it is recognised that it is 'difficult to apply sound statistics for interpretations of significance' to electron micrographs (Daly 1981).

Structurally, ascochitine belongs to the azaphilone subgroup of the quinonemethides and shows the corresponding chemical behaviour (Stossel 1981). The best documented toxin of this group is citrinin, which as well as affecting cellular permeability (Betina and Barathova 1968), inhibits algal respiration and photosynthesis (cited Damodaran *et al* 1975). Citrinin is also reported to irreversibly inhibit bovine liver glutamate dehydrogenase (GDH) (Ramadoss and Mukherjee 1977). Two distinct forms of GDH exist in higher plants (and algae); NAD and NADP linked, in mitochondria and chloroplasts respectively (Stewart *et al* 1980). 'Loss or disruption of these energy-generating systems would be expected to lead to a loss of energy-dependent properties of other membranes, especially the plasmalemma' (Wheeler 1978), conceivably resulting in endogenous ion leakage through a structurally intact but functionally inoperative cell membrane. The action of ascochitine against mitochondria and chloroplasts may be similar to that of citrinin. Citrinin also appears to uncouple

oxidative phosphorylation in dog heart mitochondria and to decrease substrate oxidation rate in rabbit liver mitochondria (Ramadoss and Mukherjee 1977).

Ascochitine is inactivated by amino acids (Oku and Nakanishi 1963) suggesting that protein binding is one mechanism of action. Citrinin is reported to bind to human and bovine serum albumin (Damodaran *et al* 1975), thus the "coagulated" appearance of organelle matrix and cellular cytoplasm may be the result of this protein binding property. 'Mechanistic interpretations of bioassay results' must however be made with caution (Yoder 1981), as it is recognised that plant cells have a limited number of responses to toxicants (Durbin 1972, Daly 1976, 1981). It has also been recognised that changes in structure can occur long after physiological effects (Hanchey 1981), so that alterations to matrix and cytoplasm texture may just be the result of membrane damage affecting cellular integrity.

Future work on the site of action of ascochitine should concentrate on studies on isolated mitochondria (oxidation rates, uncoupling of phosphorylation) and chloroplasts (effect on light and dark photosynthesis), or isolated enzymes (e.g. GDH) to elucidate the mechanism of toxicity. Ascochitine is presumably also involved in stem rot as conducting cells in leaf traces showed similar responses to ascochitine as photosynthetic cells.

Citrinin also inhibits cholesterol and ergosterol synthesis lowering liver and plasma cholesterol levels (important for control of atherosclerosis) (Endo and Kuroda 1976). While citrinin is not hepatomegalic (cf clofibrate), it is nephrotoxic (Reddy *et al* 1982, Berndt and Hayes 1982) and reportedly hepatotoxic (cited Dunn *et al* 1983). Another *Ascochyta* (*A. viciae*) metabolite, ascofuranone, has hypolipidermic properties but does not induce a hepatomegalic reaction (Sawada *et al* 1973). Trials of ascochitine to control plasma cholesterol levels would be interesting.

In the present investigation ascochitine acted as an antibiotic against *E. coli* and *B. subtilis*. These results substantiate previously reported antimicrobial activity (Bertini 1956, Oku and Nakanishi 1963, Oku and Nakanishi 1964b). Ascochitine also inhibits fungal spore germination (Bertini 1956, Oku and Nakanishi 1963, Oku and Nakanishi 1964b), and decreases submerged culture mycelial growth of a number of fungi including *A. fabae*, which was classified as an ascochitine sensitive fungus by Oku and Nakanishi (1966). If *P. clematidina* is a toxin sensitive fungus this may explain the decrease in mycelial dry weight from day 5 to day 9 as toxin concentration increased.

Ascochitine was originally proposed as a plant protectant fungicide as no phytotoxicity was observed (Bertini 1956, Oku and Nakanishi 1964b), and a wettable powder formulation controlled rice blast (*P. oryzae*) and tomato late blight (*P. infestans*) (Oku and Nakanishi 1964b). It is unlikely that an ascochitine formulation could be used to control fungal leafspot and wilt of *Clematis*.

4.7 DISEASE CONTROL AND ORNAMENTAL PATHOLOGY

One aim of this investigation was to elucidate the reason for large crop losses at New Zealand Clematis Nursery, Christchurch. Studies on *in vivo* and *in vitro* disease spread and observations of clematis propagation practices at Christchurch and Gore Bay (North Canterbury, New Zealand) suggested the main reason was cultural. Stock plants and potted cuttings were heavily leaf spotted. The grower (and probably the general public) regarded leafspots as relatively normal and unrelated to wilt. Part of the cause for this must lie with those authors in Britain who published on the disease between Gloyer's work in 1915, and 1965. The situation is still confused by authors who insist on publishing their own or others "pet theories" in widely read general publications (e.g. Harper 1977, Fisk 1986) and ignoring Gloyer's work.

The grower also disposed of dead plants on a composting heap (rather than burning) eventually recycling the potting mix without sterilisation. The grower's unsuccessful attempts to control wilt with 'Rovral' is understandable in light of results from *in vitro* fungicide trials.

Gloyer's (1915a, 1915b) original recommendations included removal of diseased leaves and vines before spraying, and ensuring vines did not matt and were adequately ventilated. Clematis growers should also avoid mechanical or wind damage to plants by correct handling and suitable growing site. Those growers who insist on good hygiene and keep to a regular spray programme only have minor crop losses (0-5%) (Steffen pers comm, Evison pers comm). Spraying with a number of fungicides ('Benlate', 'Corbel', 'Tilt', 'Bravo', 'Difolatan', 'Euparen') preferably in combinations to prevent resistance build-up, should prevent or eradicate infection.

It is difficult, even for the specialist pathologist, to keep abreast of new developments in ornamental diseases (Baker and Linderman 1979). This situation is not improved when reference books (e.g. Sutton 1980, Ellis and Ellis 1985) do not list *Ascochyta* or *Phoma clematidina* under fungi found on *Clematis*. Some popular books do list *Ascochyta* as one cause of "clematis dieback" (e.g. Buczacki and Harris 1981), usually commenting on how little is known about the disease.

There are at least 1100 genera of ornamental plants, though most are grown infrequently or rarely. Some genera include many species and the number of developed cultivars can be very high; 20 000 rose, 7 000 gladioli and 300 sweet pea varieties have been developed and named (Baker and Linderman 1979). Approximately 100 varieties of large-flowered *Clematis* are offered for sale by Fisks Nursery (Fisk 1986). All the large-flowered and some small-flowered *Clematis* are susceptible to wilt to some degree although few details are available; Lloyd (1977) identified a number of *Clematis* "more or less" resistant to wilt. Resistance to diseases has been identified in many ornamental crops (roses to powdery mildew, gladioli to *Fusarium* yellows) but the challenge of breeding for resistance is not taken up by breeders 'largely because the returns are too small' (Baker and Linderman 1979). Also, 'Benlate' offers a substantial degree of

protection to susceptible varieties (Lloyd 1977, Fisk 1986), discouraging development of breeding programmes for resistant large-flowered *Clematis*. The continued use of MBC fungicides to control wilt is probably limited as fungal resistance to benomyl arose 'surprisingly soon after it came into commercial use' in many different countries and crops (Brent 1984). Cross resistance to MBC fungicides (Dekker 1984) will prevent substitution of benomyl with thiophanate or carbendazim. A varied mixture of fungicides, good plant hygiene and cultural practices should ensure wilt free *Clematis* in the future.

CHAPTER FIVE CONCLUSIONS

The casual agent of leaf-spot and wilt of *Clematis* cultivars in New Zealand was identified as *Phoma clematidina* (Thum.) Boerema. Identification of the pathogen was based on pathogenicity towards *Clematis* cultivars and transmission electron microscopy of conidiogenesis and conidial succession. Results concurred with Boerema's (1979) decision to transfer *Ascochyta clematidina* Thumen to *Phoma* on the basis on conidial ontogeny.

Phoma clematidina is a wound pathogen, but is not a vascular pathogen. Wilt is usually the result of nodal rot following fungal ramification down the petiole from the initial leaf lesion. Leaf-spot is normally the first symptom of the wilt complex, although internodal rots and subsequent wilt have been recorded, probably the result of colonisation of stem wounds caused by kinking or wind breakage.

Five New Zealand isolates of *P. clematidina* were classified into three strains based on ten characteristics including colony morphology and growth rate, spore size, pathogenicity and fungicide sensitivity. These strains could not be be identified as, or consistently related to type or herbarium specimens.

Pathogenicity is associated with *in vitro* production of the toxin ascochitine. This toxin is partially responsible for symptom production causing leaf disc blackening similar to that observed in natural lesions, and induces endogenous electrolyte leakage from sensitive *Clematis* leaf tissue. Ascochitine is probably involved in rot of vascular tissues prior to wilt. Correlations between toxin insensitivity and wilt resistance are affected by hybrid ability to abscise or senesce infected leaves. The toxin sensitive but wilt resistance cultivar 'Huldine' prevents fungal colonization of the petiole by leaf senescence. The toxin insensitive, wilt resistant cultivar 'Montana' abscises infected leaves before the hyphae reach the abscission zone and petiole. Toxin sensitive, wilt susceptible cultivars do not abscise or senesce infected leaves.

Ascochitine was identified by ^{1}H NMR spectroscopy, after isolation and purification from *P. clematidina* liquid cultures and reverse phase HPLC analysis for preparation purity. Identification was verified by ^{13}C NMR spectroscopy, and electron impact and chemical ionisation mass spectroscopy. Ascochitine was also isolated from leaf lesions. Transmission electron microscopy suggested chloroplasts and mitochondria were the cellular sites of toxin action, although damage to the nucleus and other cellular constituents was apparent.

A wide range of protective and systemic fungicides were assessed for *in vitro* fungitoxic activity. The most effective fungicides from each group (chlorothalonil and fenpropimorph) were combined and utilised in a glasshouse trial. This mixture demonstrated good disease control although further development of mixture ratios, concentrations and spray periods is necessary. A number of other fungicides including

the MBC fungicides, propanicazol, prochloraz, dichlorofluanid and capatafol are also recommended for glasshouse trials.

Cultural practices (good plant hygiene and care, correct disposal of diseased material and selection of sheltered growing sites) will remain important means of controlling this disease, while the use of fungicides will also contribute to disease control.

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APPENDIX 1 CULTURE MEDIA

GA Glucose agar

2g D-glucose, 8g BBL nutrient broth, 15g agar, 1000ml distilled water

GSP Glucose-strephomycin-penicillin agar

GA and penicillin-G 300 *u*g/ml, streptomycin sulphate 130 *u*g/ml

nPDA (natural) Potato dextrose agar

200g washed cubed old potatoes, boiled 1h, squeezed throught fine muslin, added to 20g dextrose, 20g agar, distilled water to 1000ml. Autoclave 20psi 15min

OA Oatmeal agar

30g oatmeal boiled 1h, squeezed through fine muslin, distilled water to 1000ml, add 20g agar. Autoclave 20psi 15min

APPENDIX 2 FIXATIVES AND FIXATION PROCEDURES

Optical Microscopy

FAA Formalin aceto alcohol

Formaldehyde 30ml, glacial acetic acid 50ml, ethanol 315ml, distilled water 605ml

(a) Fixation

Specimens were fixed in FAA for a minimum of 24h.

(b) Dehydration

TBA (tertiary butyl alcohol) series

50% TBA 1h, 70% TBA overnight, 85% TBA 1h,

95% TBA 1h, 3 changes 100% TBA 1h each at 40°C,

100% TBA overnight at 40°C.

(c) Infiltration

50:50 TBA: liquid parafin 1 day 40°C, infiltrating

wax ('Paraplast') 1 day 70°C, 2 changes 'Paraplast' in 70°C vacuum oven, 1 day each.

(d) Embedding

Embedded in 'Paraplast' and cooled under water for 4h.

Transmission Electron Microscopy

(a) Fixation

Specimens were fixed in 3% (v/v) glutaraldehyde in 0.075M phosphate buffer[1] for 3h under vacuum, washed in 3 changes of buffer for 1h, then post-fixed in 1% (w/w) OsO_4 in buffer for 3h.

(b) Dehydration

OsO_4 was replaced with buffer for 15min, then specimens were dehydrated in acetone at 20% stages for 15min each. Three changes of 100% acetone for 20min each preceded infiltration.

(c) Infiltration

Acetone was replaced by 25:75 (v/v) Spurr's resin : acetone, and specimen vials rotated for 4h. This mixture was replaced by 75:25 mixture and vials left overnight on the rotator.

(d) Embedding

Specimens were embedded in 100% Spurr's resin, which was polymerised at 70^{O}C for 24h.

Note[1]

0.075M Phosphate buffer pH 7.2

Buffer A 0.2M $Na_2HPO_4.2H_2O$ 17.805g/ 500ml

Buffer B 0.2M NaH_2PO_4 15.605g/ 500ml

36ml A + 14ml B + 83ml distilled water

APPENDIX 3 STAINS

Pianeze *III*B

Malachite green, 1% (w/w) aq. solution, 50ml
Acid fushsin, 1% (w/w) aq. solution, 10ml
Martius yellow, 1% (w/w) aq. solution, 1ml
Distilled water, 89ml
95% ethanol, 50ml

Staining Procedure

(1) Dewax with 3 five min changes xylol
(2) Rinse 100% ethanol
(3) 5min 95% ethanol
(4) 5min 70% ethanol
(5) 5min 50% ethanol
(6) 2h Pianeze III_B
(7) Rinse 95% ethanol
(8) 1min 95% ethanol
(9) 30s acid alcohol[1]
(10) Rinse 95% ethanol
(11) 1min 100% ethanol
(12) Clear in Clove oil
(13) Xylol wash

Note

Best results occurred with 1% (v/v) glacial acetic acid in 95% ethanol (Gurr 1965), rather than 95% ethanol plus 2 drops of HCl (Simons and Shoemaker 1952*).

10% Lacto-cotton blue

Lactophenol (anhydrous), 78.39ml
Distilled water, 2.00ml
Cotton blue, 0.01g

Azur *II*

50:50 (v/v) Azur blue: methylene blue mixed immediately prior to use.

Pianeze IIIB staining of Spurr's sections

Sections heat fixed to glass slides, then covered with drop of sodium ethoxide (saturated solution of NaOH in ethanol) for 2 minutes, washed with 100% ethanol and stained (from step 2)

* Simons, S. A. and Shoemaker, R. A. (1952)
Differential staining of fungus and host cells using a modification of Pianeze III_B
Stain Technology 27(2): 121

APPENDIX 4 ADDRESSES

Herbariums

CMI Commonwealth Mycological Institute
Ferry Lane, Kew, Surrey TW9 3AF, England

CUP Plant Pathology Herbarium
Cornell University, Ithaca, New York 14853, U. S. A.

PAD Univesita Di Padova
Istituto Di Botanica E Fisiologia Vegetale,
Via Orto Botanico 15, 35100 Padova, Italy

Correspondence

Mr R. J. Evison
Managing Director, Treasures of Tenbury Limited,
Burford House, Tenbury Wells, Worcestershire WR15 8HQ, England

Mr J. Fisk
Fisk's Clematis Nursery, Westleton, Saxmundham,
Suffolk 1P17 3AJ, England

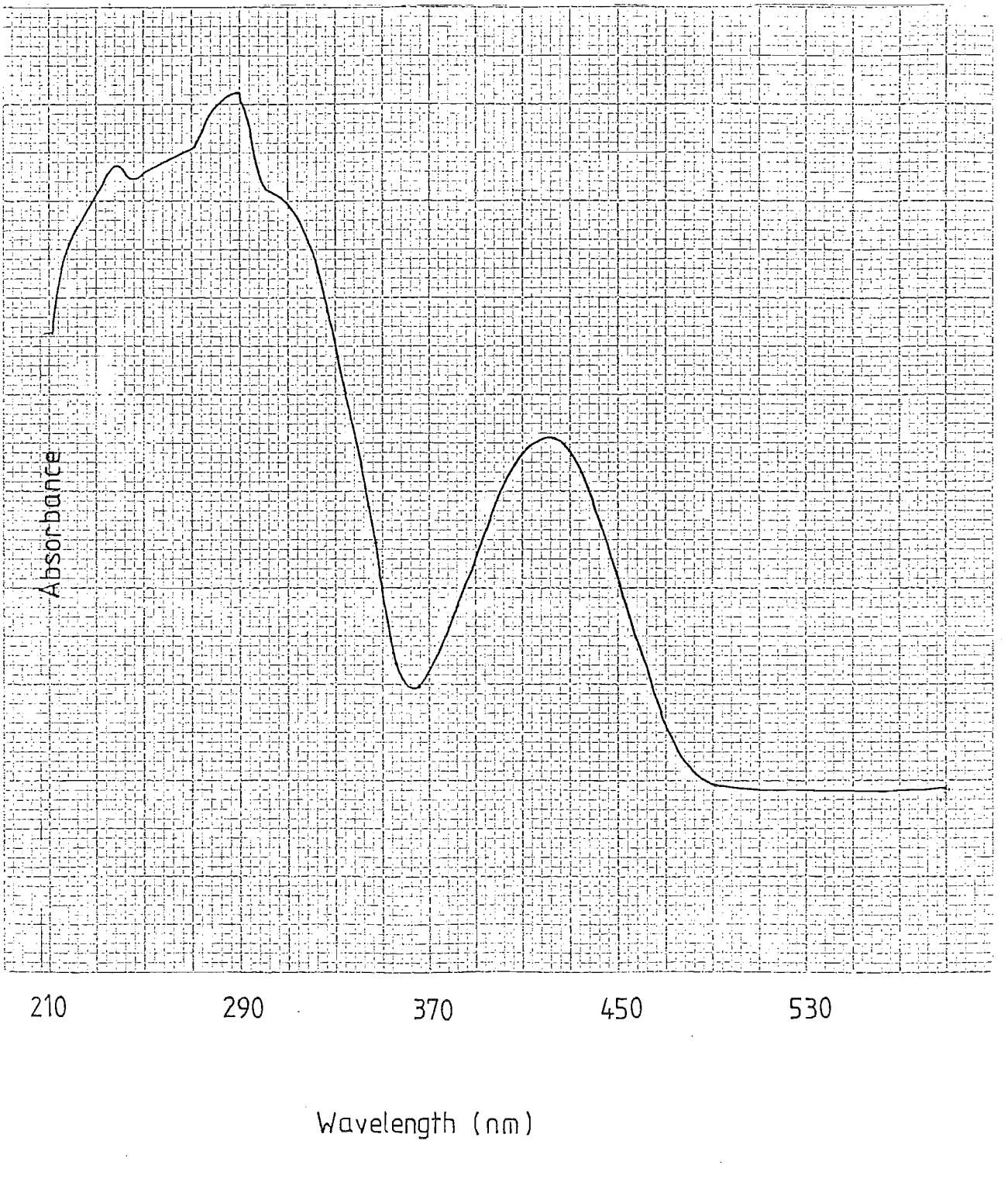
Mr A. Keay
New Zealand Clematis Nurseries, 67 Ngaio Street,
St. Martins, Christchurch, New Zealand

Mr D. Lear
New Zealand Clematis Nursuries, Gore Bay, R. D. 3, New Zealand

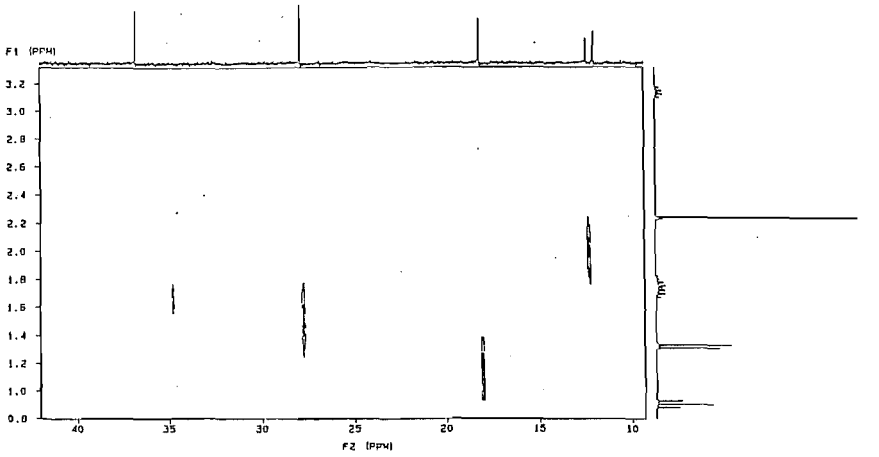
Mr A. H. Steffen
Aurther H. Steffen Inc., Wholesale Nursery, 1259 Fairport Road,
P. O. Box 184, Fairport, New York 14450, U. S. A.

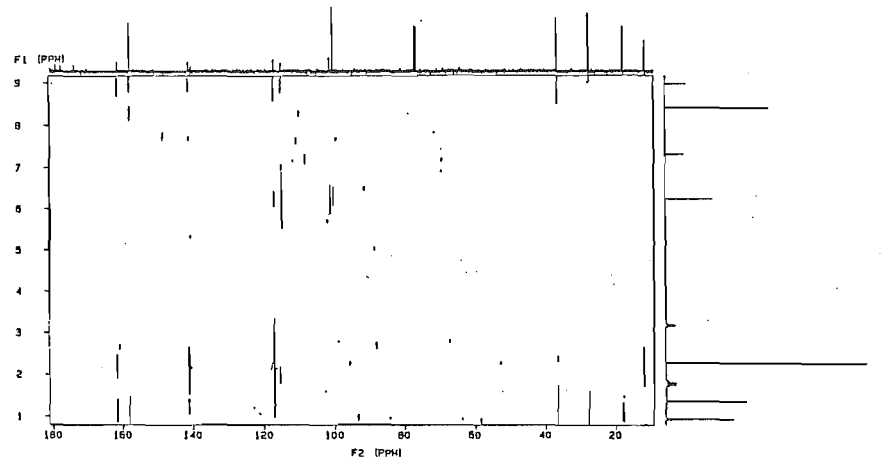
Sunshine Environmentals
Roberts Line, P. O. Box 5029, Palmerston North, New Zealand

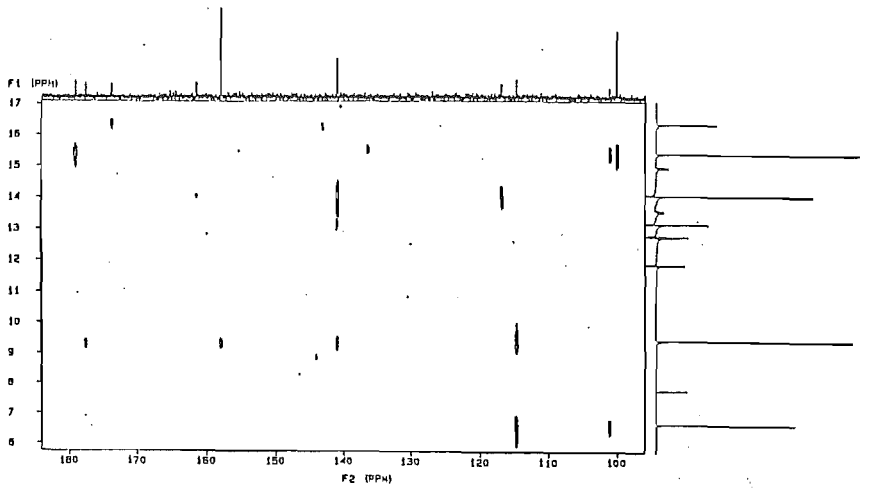
Appendix 5 Toxin ultraviolet spectrum after 24h in methanol



Appendix 6 Toxin heteronuclear correlations







APPENDIX 7 ISOLATE RELATIONSHIPS

Isolates followed by the same letter in the same row are equivalent at p=0.05 (DNMRT)

Characteristic	Isolate association				
Appearance	EM	RC a b	LB a b	HD b	MT
Growth	EM	RC a	HD a	LB	MT
Spore length	RC a	EM a	HD a	LB a	MT
Spore width	EM a	HD a	RC a	LB	MT
Aggressiveness	EM a	RC a	LB a	HD a	MT
Virulence	HD	LB a	RC a	EM	MT
Toxin prodn.	EM a	LB a	HD a	RC	MT
Mycelium	HD a	RC a	LB a	EM b	MT b
'Bravo'	EM	RC a	LB a	HD a	MT
'Corbel'	LB	EM a	RC a	HD	MT

Isolates were classified into strains based on number of different and equivalent (DNMRT p=0.05) characteristics or responses.

APPENDIX 8 TOXIN WEIGHT CORRELATIONS

Crude ascochitine was isolated from infected leaf discs at *c* 4x the concentration necessary to induce *in vitro* electrolyte leakage, suggesting the assay was a substantially accurate model of *in vivo* pathogenicity.

Toxin isolated from leaf discs lesions: 0.63mg/ g leaf tissue
Lesions size on 'Lady Betty Balfour' leaf dics: 10.1cm
Assuming infected and healthy tissue are equivalent weights then, 1.58mg toxin/ 1g infected tissue

Weight of leaf tissue for toxin assay: 0.18g
Weight of toxin used (10ml x 5 *u*g/ml): 50*u*g
Weight for weight: 278 *u*g/ 1g (0.278 mg/g) leaf tissue

APPENDIX 9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DEFINITIONS

k'

Capacity ratio of mobile phase. This ratio ($k' = (V_1-V_0)/ V_0$) is idicative of the retention and resolution of a compound. Usually k' is changed by varying the solvent strenght. A k' value in the range 2-6 tends to optimize resolution (Phillips *et al* 1980).

t'

The t' value reflects peak tailing. Calculated by dividing the total peak width minus the width of the tail by the total peak width. A peak with no tailing has t' = 1.0 (Phillips *et al* 1980).

V_0

Void Volume. Dead volume that must be eluted before injected sample solvent/ elutant can reach detector.

V_1

Volume eluted before sample detected.